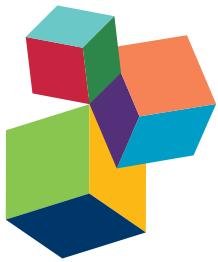


GRAPPLING WITH THE MULTIFACETED WORLD OF THE DNA DAMAGE RESPONSE

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GRAPPLING WITH THE MULTIFACETED WORLD OF THE DNA DAMAGE RESPONSE

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In martial arts, the grappling defines a hand-to-hand combat. Scientists all over the world every day engage in a scientific struggle to reveal and solve an intricate and entangled network of repair pathways, referred to as DNA Damage Response, which represent the ultimate bulwark to protect DNA from genotoxic injuries.

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DNA damage is a major threat to genomic integrity and cell survival. It can arise both spontaneously and in response to exogenous agents.

DNA damage can attack most parts of the DNA structure, ranging from minor and major chemical modifications, to single-strand breaks (SSBs) and gaps, to full double-strand breaks (DSBs). If DNA injuries are mis-repaired or unrepaired, they may ultimately result in mutations or wider-scale genome aberrations that threaten cell homeostasis. Consequently, the cells elicit an elaborate signalling network, known as DNA damage response (DDR), to detect and repair these cytotoxic lesions.

This Research Topic was aimed at comprehensive investigations of basic and novel mechanisms that underlie the DNA damage response in eukaryotes.

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Editorial: Grappling with the Multifaceted World of the DNA Damage Response

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Keywords: DNA damage, DNA damage response (DDR), DNA repair pathways

The Editorial on the Research Topic

Grappling with the Multifaceted World of the DNA Damage Response

DNA is the repository of the genetic information in each living organism and its integrity and faithful transmission has to be ensured across generations for our own survival. Despite DNA has evolved as a more stable molecule than its ancient predecessor RNA, it is not able to guarantee life-long stability. Random changes occurring at the level of DNA represent the main source of genetic variability and the raw material on which Darwinian evolution acts.

In all organisms, cells experience massive amounts of damaging events each day. For instance, DNA injuries occur with a frequency of 10^4 – 10^6 in a single human cell per day. DNA lesions can have deleterious effects, as they interfere with basic cellular transactions, such as genome replication and transcription. If DNA injuries are mis-repaired or unrepaired, they may ultimately result in mutations or wider-scale genome aberrations that threaten cell homeostasis. As a proof of the fact, genome instability is a hallmark of tumorigenesis and tumor progression (Hanahan and Weinberg, 2011). On the other hand, DNA injuries increase during time as result of the imbalance between generation and scavenging of by-products deriving from cellular metabolism. Therefore, DNA damage promotes cellular senescence or cell death (Malaquin et al.), thus contributing to aging or to onset of aging-related disorders.

DNA damage can attack most parts of the DNA structure, ranging from minor and major chemical modifications, to single-strand breaks (SSBs) and gaps, to full double-strand breaks (DSBs; Brown and Baltimore, 2000). DNA lesions can arise as consequence of physiological processes like DNA replication (Jossen and Bermejo; Ouyang et al.) or can be caused by the exposure to environmental toxins. For example, the mis-incorporation of nucleotides during DNA replication contributes to the spontaneous mutation rate in an organism. While, canonical DNA polymerases are proofreading enzymes able to recognize and correct many of these errors, some mutations can escape this process. Other endogenously-arising DNA alterations lead to loss or modification of DNA bases (Lindahl, 1993). By-products of physiological cellular metabolism, such as reactive oxygen species (ROS) derived from oxidative respiration (Markkanen et al.), side-products of lipid peroxidation, or aldehyde metabolism (Finkel and Holbrook, 2000), constitute a permanent enemy to DNA integrity as they ultimately lead to DNA oxidation and breaks.

DNA damage is otherwise produced environmentally by chemical and physical sources. The most pervasive DNA-damaging agents are ultraviolet (UV) light derived from sunlight and ionizing radiation (IR). Despite the ozone layer absorbing the most dangerous part of the ultraviolet spectrum (UV-C), the other two types of UV radiation, UV-A and UV-B, are able to penetrate Earth's atmosphere and reach the planet's surface, thus being of greatest concern to humans. Exposure to UV radiation induces formation of cyclobutane pyrimidine dimers (CPDs) and 6–4

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photoproducts. Such lesions distort DNA's backbone, introducing bends or kinks that can represent a serious impediment for transcription and replication processes. In addition, IR originating from the decay of naturally occurring radioactive compounds or from medical treatment employing X-rays and/or radiotherapy, also generates various forms of DNA damage. Finally, certain types of chemicals can also cause a variety of DNA lesions. DNA-damaging chemicals are mainly used in chemotherapy, but can be present in contaminated foods, such as heterocyclic amines produced in over-cooked meat or aflatoxins detected in contaminated peanuts. Remarkably, tobacco products derived by cigarette smoking are the most prevalent environmental DNA-damaging chemical agents as they cause a wide variety of DNA adducts and oxidation, which can ultimately trigger cancer of the lung and adjacent tissues.

In order to preserve the integrity of the genome, cells have evolved an integrated signaling network of damage detection and repair: the DNA damage response (DDR; Lindahl and Barnes, 2000). The DDR senses different types of genotoxic lesions and mounts coordinate and multi-faceted responses, that ultimately fix DNA lesions in a timely manner and prevent their conversion into permanent genome mutations (Hoeijmakers, 2001; Harrison and Haber, 2006; Harper and Elledge, 2007; Jackson and Bartek, 2009). Moreover, the DDR also activate checkpoints to arrest or delay cell cycle progression or, if repair fails, trigger apoptosis. Cell cycle checkpoints are a genome surveillance mechanisms monitoring and controlling the timing and order of cell cycle events (Ferretti et al.; Jossen and Bermejo). Indeed, the DDR signaling pathways modulate the activity of cell cycle regulators and DNA repair enzymes, thus ensuring tight coordination of DNA repair with cell cycle progression. At molecular level, DDR is organized into an elaborate network of interacting pathways, the constituents of which can be grouped into three major classes of proteins that act in concert to translate signals of damaged DNA into appropriate downstream responses. These comprise (1) sensor proteins that recognize abnormally structured DNA and initiate the signaling response, (2) transducers factors that relay and amplify the damage signal on the surrounding chromatin structure, and to (3) effector proteins that ultimately lead to DNA damage repair (Bartek and Lukas, 2007; Harper and Elledge, 2007). Thus, the DDR necessitates to be spatiotemporally regulated (Ferrando-May et al.) because, if misused, it can wreak havoc on DNA integrity.

The wide diversity of DNA injuries requires the activation and cooperation of multiple and largely distinct DNA repair mechanisms (Stracker et al.). Different DNA damages are repaired by a sequence of catalytic events mediated by a plethora of enzymes. Currently DNA repair pathways can be grouped in different categories.

Nucleotide Excision Repair (NER), involves global genome repair (GGR) and transcription-coupled repair (TCR), recognizes and repair helix-distorting base lesions, such as pyrimidine dimers, induced by UV light. A key aspect of NER is the excision of the damaged DNA by specific endonuclease as a short oligonucleotide, thus leading to the formation of single-strand DNA, which is then acted by DNA polymerases before ligation occurs. In Base Excision Repair (BER), a non-helix

distorting base modification, such as oxidation or alkylation, is recognized by a DNA glycosylase that initiates the excision of the modified base, thus leaving an apurinic or apirimidinic site, from which nuclease, polymerase and ligase enzymes can complete the repair. This pathway can operate via two sub-pathways, short-path (SP-BER) or long-path (LP-BER), based on the length of DNA re-synthesis. However, these two pathways often converge and cause the formation of a SSB, which is in turn sealed by a rapid process dependent on PARP [Poly (ADP-ribose) polymerase]-mediated signaling. In mismatch repair (MMR; Bak et al.; House et al.) incorrect polymerase proofreading or ribonucleotide mis-incorporation in the DNA chains occurring during DNA replication triggers the activation of post-replicative DNA repair machinery, which degrades mis-paired nucleotide of the newly synthesized strand, thus assisting DNA polymerases with another chance to generate an error-free copy of the template sequence (Jiricny, 2013). Notably, lesions that block replication forks progression are often by-passed by DNA damage tolerance (DTT) pathways. Specialized translesion synthesis (TLS) DNA polymerases, harboring a less stringent base-pair requirements than replicative polymerases, restart stalled replication forks, thus preventing their collapse and the consequent DSB formation, but at the expense of a higher mutation rate; DTT pathway promotes the completion rather than the accuracy of DNA replication. Repair of DSBs relays on two major pathways: non-homologous end joining (NHEJ; Lieber, 2008) and homologous recombination (HR; San Filippo et al., 2008). Whereas, NHEJ can operate throughout the cell cycle and is mostly used by post-mitotic cells, HR requires the presence of an undamaged homologous template, usually a sister chromatid, to mediate faithful repair and is restricted to S- and G2-phase of actively replicating cells. NHEJ promotes direct ligation of the two ends flanking the DSB without the need of extensive DNA-end processing. However, small insertions, deletions, and substitutions occurring at the break site, make NHEJ an error-prone process. On the contrary, HR mainly ensures an accurate repair of DSBs (Ferretti et al.; Guirouilh-Barbat et al.) as it uses the homologous chromosome as a template and it is often dedicated to fix breaks arising from DNA replication stress. HR requires the generation of ssDNA by DNA-end resection, which in turn invades the undamaged template leading to the formation of branched DNA structures. Therefore, DNA synthesis and recombination intermediate dissolution complete the HR-mediated repair process. Furthermore, DSBs which harbor a complementary flanking sequence can also be repaired by alternative end-joining (alt-NHEJ) also called microhomology-mediated end-joining (MMEJ), when microhomologies are present, or via single-strand annealing (SSA) when longer repeats are present (Decottignies). Although, MMEJ and SSA also rely on DNA-end resection reminiscent of HR, they can lead to loss of intervening sequence and thus are highly mutagenic (Kalan et al.; Guirouilh-Barbat et al.; Blanco and Matos). Lastly, DNA interstrand crosslinks (ICLs) represent the most serious kind of DNA lesions, as they must be repaired through a complex mechanism involving NER, TLS and HR, which are coordinated by the Fanconi Anemia (FA) pathway.

Spatiotemporal recruitment of DDR factors to sites of DNA damage is promoted by sensor proteins, which activate specific signaling cascades. It is becoming increasingly clear the biological relevance of chromatin structure and epigenetic marks in the DDR orchestration (Ferrando-May et al.; House et al.; Savic). Efficient repair of DNA damage is complicated by the fact that DNA is packaged into a condensed structure. Then, to facilitate access of the DNA repair machinery to the lesion, transient rearrangement of the chromatin has to occur. The nucleosome is the fundamental unit of the chromatin and consists of core particle, in which DNA is wrapped around a histone octamer. Various histone posttranslational modifications (PTMs) such as methylation, phosphorylation, acetylation, sumoylation, and ubiquitination have been reported at different amino acid residues of histones (Bartocci and Denchi; Bologna and Ferrari; Ouyang et al.; Pinder et al.; Vaz et al.). Thus, the large number of histone PTMs and the existence of diverse histone variants can define specific chromatin configurations, which characterize distinct stages in the DDR. Emerging evidences suggest that non-coding RNAs (ncRNAs), as master regulators of chromatin, can control the activation of DNA repair machinery by promoting chromatin organization in different epigenetic states. On the other side, ncRNAs, like microRNAs, may also act in the biogenesis of core protein-coding components of DDR pathways (Boucas et al.; Montecucco and Biamonti).

DDR regulates several physiological processes. DNA-repair enzymes can introduce physiological DSBs to promote genetic variability during meiosis in germ cells. DDR is indeed required to promote genetic diversity via sexual reproduction by ensuring the exchange of genetic information between homologous chromosomes before meiosis (Carroll and Marangos). Moreover, recombination processes are involved in the maturation of the immune system, such as class-switch and V(D)J recombination in B- and T-lymphocytes and play a critical role in the activation of immune surveillance and in generating immune-receptor diversity. Finally, DDR can determine whether a cell undergoes apoptosis or terminal differentiation through senescence. In this regard, markers of unrepaired DSBs accumulate with age at telomeres (Arnoult and Karlseder, 2015; Feuerhahn et al., 2015), which are nucleoprotein structures located at the end of our chromosomes. Due to the inability of the replication machinery to fully replicate chromosomal ends, telomeres shorten with each cell division until they hardly retain telomeric DNA repeats that are instead recognized as DSBs (Rosen; Henriksson and Farnebo). Thus, under such context of chronic DDR activation at telomeres, cells can enter into apoptosis or senescence (Fumagalli and d'Adda di Fagagna, 2009).

Considering the biological relevance of DDR in diverse physiological settings, “inherited” DDR defects predispose cells to genome instability and consequent diseases, like neurodegenerative disorders, immune deficiencies, infertility, age-related pathologies, cardiovascular diseases, metabolic syndromes, and cancer. The DDR is usually activated in precancerous cells experiencing oncogene-induced replicative stress and can be considered as an anticancer barrier that protects against full cellular transformation. Otherwise, targeting the replicative surge of cancer cells and their DDR/checkpoints

unbalance are the basis for classical radio- and chemo-therapy and impairment of DNA repair pathways may represent a window for therapeutic opportunity (Shahbazi et al.; Kotsinas et al.). Cancer cells displaying specific DNA repair defects become “addicted” to complementary, but often inaccurate repair pathways in order to fuel their unscheduled expansion. Recently, this effect has been successfully exploited for synthetic lethality strategies, where small molecule inhibitors of these alternative pathways lead to selective killing of cancer cells harboring a specific genetic background, as in the case of PARP inhibitor treatment of HR-deficient tumors. Although, DDR components represent attractive targets for the development of novel cancer-therapies, they can also provide a common mechanism for cancer-therapy resistance. The development of diagnostic procedures to identify DDR components altered during oncogenesis might allow effective detection of pre-malignant diseases and tailor DNA-damaging or DDR inhibitor therapies for individual patients.

The relevance of the chemistry and biology of the DDR was underscored by the Royal Swedish Academy of Sciences when it awarded the Nobel Prize in Chemistry 2015 to three pioneering scientists—Thomas Lindahl, Paul Modrich, and Aziz Sancar—for having, independently of each-other, mapped and elucidated the molecular basis of how cells repair their DNA. In the early 1970s, Thomas Lindahl was the first scientist to demonstrate that the DNA decays at a slow but noticeable rate. This insight led him to quest for repair enzymes discovering in this way the BER pathway (Lindahl, 1974). At the same time, Aziz Sancar investigated the effects of UV radiation on bacteria leading him to uncover the NER pathway (Sancar and Rupp, 1983). Paul Modrich instead focused his research on DNA replication finding out how cell corrects errors during cell divisions: the mismatch repair mechanism (Lahue et al., 1989). In the last two decades, oncology research has been building on those findings to develop the aforementioned conventional DNA-damaging cancer treatment as well as newer targeted therapies by inhibiting repair pathways.

This Research Topic is aimed at comprehensive investigations of basic and novel mechanisms that underlie DNA damage response in eukaryotes. All authors in this Research Topic have provided their broad perspectives on distinct aspects of DDR and their insightful thoughts will benefit the field and will provide fertile ground for future investigations that we look forward to seeing develop.

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DDR-mediated crosstalk between DNA-damaged cells and their microenvironment

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The DNA damage response (DDR) is an evolutionarily conserved signaling cascade that senses and responds to double-strand DNA breaks by organizing downstream cellular events, ranging from appropriate DNA repair to cell cycle checkpoints. In higher organisms, the DDR prevents neoplastic transformation by directly protecting the information contained in the genome and by regulating cell fate decisions, like apoptosis and senescence, to ensure the removal of severely damaged cells. In addition to these well-studied cell-autonomous effects, emerging evidence now shows that the DDR signaling cascade can also function in a paracrine manner, thus influencing the biology of the surrounding cellular microenvironment. In this context, the DDR plays an emerging role in shaping the damaged tumor microenvironment through the regulation of tissue repair and local immune responses, thereby providing a promising avenue for novel therapeutic interventions. Additionally, while DDR-mediated extracellular signals can convey information to surrounding, undamaged cells, they can also feedback onto DNA-damaged cells to reinforce selected signaling pathways. Overall, these extracellular DDR signals can be subdivided into two time-specific waves: a rapid bystander effect occurring within a few hours of DNA damage; and a late, delayed, senescence-associated secretory phenotype generally requiring multiple days to establish. Here, we highlight and discuss examples of rapid and late DDR-mediated extracellular alarm signals.

Keywords: DNA damage response, senescence, bystander effect, senescence secretome, inflammation, microenvironment, tissue damage

The DNA damage response (DDR) signaling network is essential in the maintenance of genomic stability, via the initiation and coordination of DNA repair mechanisms with appropriate cell cycle arrest checkpoints (d'Adda di Fagagna, 2008; Jackson and Bartek, 2009). The DDR is initially propagated by a series of effective and rapid post-translational modifications culminating in the activation of nodal transcription factors like p53, which organize additional DDR transcriptional responses (Harper and Elledge, 2007).

Briefly, a typical DDR cascade begins with the recruitment and activation of an apical DDR kinase like ATM (ataxia-telangiectasia mutated) to DNA double-strand breaks (DSBs) by damage sensors such as the MRN complex (MRE-11, Rad-51 and NBS-1 proteins). This leads to the local phosphorylation of multiple ATM substrates in the chromatin surrounding the DNA lesion,

almost always including the histone variant H2AX (phospho-H2AX or γH2AX). These local chromatin modifications provoke the further recruitment of additional DDR mediators at the break, including 53BP1 and MDC1, which amplify chromatin modifications over megabases of DNA generating macroscopic structures called DNA damage foci (DDF; Rogakou et al., 1998; Bonner et al., 2008) that allows for the direct visualization of single DSBs in mammalian cell nuclei (Rogakou et al., 1999). Simultaneously, the distal propagation of the DDR signal within the cell promote cell cycle checkpoints and the activation of p53 (Rodier et al., 2007). When DNA lesions are repairable, the ensuing growth arrest is transient, eventually resulting in cell cycle resumption, and a return to normality. In contrast, severe or irreparable DNA lesions trigger prolonged DDR signaling, resulting in apoptosis or senescence (permanent growth arrest; Campisi and d'Adda di Fagagna, 2007).

The DDR Generates Extracellular Signals

The DDR is mostly known for its role as a cell-autonomous, intracellular signaling cascade that regulates DNA repair and cell cycle checkpoints. However, in the context of higher organisms with multicellular tissues, cells have developed intricate intercellular communication mechanisms that the DDR employs to trigger extracellular alarm signals. Conceptually, it is entirely plausible that damaged cells can signal to other cells that their genome has been compromised, essentially generating tissue-wide stress responses. In fact, these DDR-mediated extracellular alarm signals can be subdivided into at least two waves: rapid

and late. While we are still far from a complete understanding of extracellular DDR signaling, it is already well established that specific communication mechanisms including cell surface bound and soluble molecules are involved in this process (Figure 1). Bystander responses received by cells adjacent to damaged cells have been described, and more importantly, some soluble signals have been proposed to travel further in the body, creating additional potential therapeutic intervention opportunities (Tchkonia et al., 2013; Havaki et al., 2014).

A Rapid Extracellular DDR Signal Reaches Undamaged Bystander Cells

Accumulating experimental evidence shows that damaged cells rapidly transmit a DDR-dependent stress signal to neighboring healthy cells, provoking paracrine activation of stress responses such as a bystander DDR. While not originally linked to the DDR itself, this phenomenon was first described under conditions in which only 1% of the cells in a population were irradiated by a low dose of alpha-particles, yet 30% of the cells exhibited chromosomal changes (Nagasawa and Little, 1992). This bystander damage response could be an important mechanism used to rapidly amplify the effect of low dose irradiation by transferring DNA-damage signals from irradiated cells to non-irradiated ones.

It is now clear that non-irradiated cells can adopt common DNA damage-associated phenotypes from adjacent irradiated cells, including micronuclei formation, altered expression of stress-related genes, various epigenetic changes, increased frequency of mutations, induction of apoptosis or senescence, and even malignant transformation (Azzam et al., 2002; Nagasawa and Little, 2002; Morgan, 2003; Ko et al., 2006). Interesting mechanistic evidence supporting the activation of the

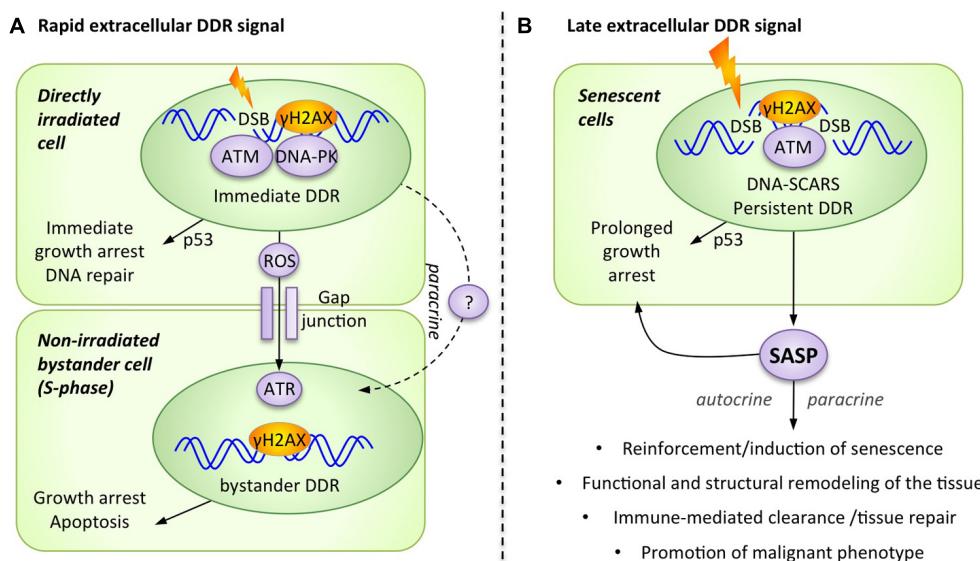


FIGURE 1 | The DNA damage response (DDR) generates alarm signals that are transmitted from the DNA-damaged cell to the extracellular microenvironment. **(A)** Rapid extracellular DDR signals occur in response to DNA damage and are transmitted to neighboring

cells via direct cell-cell contact and paracrine signals. **(B)** Late extracellular DDR signals occur in response to persistent DNA damage signaling and are collectively known as the senescence-associated secretory phenotype (SASP).

DDR in bystander cells includes the formation of DNA damage foci (DDF), which also suggests the accumulation of DSBs in these cells (**Figure 1A**; Sokolov et al., 2007). The formation of bystander γH2AX foci has been observed in a number of experimental systems, including human cultured cells and three-dimensional tissue models, as well as *in vivo* mouse models (Sokolov et al., 2005; Sedelnikova et al., 2007). Furthermore, normal fibroblasts that were exposed to damaged cells, either directly through co-culture or indirectly through conditioned media, demonstrated many other typical DDR markers in DDF, including 53BP1, phospho-ATM, and the focal presence of the ATM-activating MRN complex (Sokolov et al., 2005; Sedelnikova et al., 2007).

The pathways involved in the transmission of alarm signals generated by irradiated cells remain ill defined, but emerging insight appears promising. For example, the activation of DNA-PKcs and ATM is necessary for the generation of a bystander signal from the damaged cell, but these kinases are not required for signal reception in non-irradiated bystander cells (Hagelstrom et al., 2008). Alternatively, the kinase ATR could be required in the recipient bystander cell to allow for the formation of DDF (containing γH2AX, 53BP1, BRCA1) and the subsequent activation of ATM. Importantly, this ATR-dependent bystander DDR activation occurs only in S-phase cells, consistent with the concept that replication stress is a major trigger for ATR activation (Burdak-Rothkamm et al., 2007, 2008). Accordingly, the radiation-triggered extracellular alarm signal preferentially affects non-irradiated cells that display high rates of replication and transcriptional activities (Dickey et al., 2012). Overall, this suggests that not all bystander cells equally trigger a bystander DDR, and that actively dividing cells are most receptive to this signal (**Figure 1A**).

Two distinct pathways for the transmission of rapid extracellular DDR signals have been proposed: direct cell-cell communication and paracrine interaction (**Figure 1A**). For cells in direct physical contact, small molecules (<1.5 kDa) are usually transmitted through multimeric protein channels termed gap junctions, and the rapid extracellular DDR signal is effectively abrogated following the use of pharmacological inhibitors against gap junctions (i.e., lindane) or by the genetic ablation of an essential gap junction component, connexin 43 (Azzam et al., 1998, 2001). To directly communicate with neighboring cells, the DDR has also been shown to increase the presence of selected cell surface ligands and receptors on damaged cells. For example, some DDR regulated cell surface molecules can subsequently engage surrounding immune cells (NKG2D ligands) or can influence damaged cells survival (DR5 receptor) via receptor-ligand engagement (Wu et al., 1997; Finnberg et al., 2005; Gasser et al., 2005; Lam et al., 2014). A second signaling route consists of the release of soluble factors into the extracellular media, which act in a paracrine manner to stimulate neighboring cells. Consistent with this mechanism, the addition of conditioned media from irradiated cells is sufficient to induce DDF and bystander DDR activation in non-irradiated cells (Sokolov et al., 2005; Shao et al., 2008; Dickey et al., 2009; Klammer et al., 2010).

The molecular players directly tasked with conveying rapid stress signaling from cell to cell are still poorly defined. The

most commonly described family of factors is reactive oxygen or nitrogen species (ROS/NOS), produced at high levels in the damaged cell (Havaki et al., 2014). Indeed, the activation of the DDR as well as its downstream phenotypes in bystander cells (i.e., up-regulation of stress genes, micronucleus formation) is suppressed by superoxide dismutase activation or by ROS inhibitors (Azzam et al., 2002; Little et al., 2002). ROS, and in particular H₂O₂, which has a relatively longer half-life, can freely diffuse across plasma membranes or through gap junctions, causing DNA damage at distant sites (Azzam et al., 2003). Oxidative stress can result in DNA lesions in the form of single strand DNA breaks (SSBs) that can be converted to DSBs when unresolved or abundant, suggesting that ROS can account for at least a subset of the observed bystander DNA damage events (Tanaka et al., 2006). The second class of soluble factors involved in long distance extracellular DDR signaling includes molecules such as transforming growth factor-β1 (TGF-β1) and tumor necrosis factor-α (TNF-α; Iyer et al., 2000). In addition to its direct role in signaling, the TGF-β1 secreted by the irradiated cells also contributes to the intracellular increase of ROS and NOS in bystander cells, most likely through NAD(P)H oxidase activation (Burdak-Rothkamm et al., 2007, 2008; Shao et al., 2008). Some, and perhaps most, rapid intercellular damage signaling processes also play a role in the late extracellular response (see below). However, the opposite is not necessarily true, for example, cytokines like IL-6 and IL-8 are exclusive to the late phase following irradiation (Rodier et al., 2009).

A Late Senescence-Associated Extracellular DDR Signal Modifies the Microenvironment

In general, the early phase of the intracellular DDR signaling cascade is a well-established response to nuclear damage, occurring within seconds to hours of the initial assault. But when DNA lesions are particularly severe or irreparable, such as uncapped telomeres (d'Adda di Fagagna et al., 2003), the DDR signal can persist and provoke programmed cell death (apoptosis) or permanent growth arrest (cell senescence; Rodier and Campisi, 2011). While apoptotic cells are rapidly eliminated, damaged senescent cells can persist for extended periods and accumulate in damaged or aging tissues (Baker et al., 2011). Senescence typically depends on the p53/p21 and p16INK4a/RB tumor suppressor pathways (Campisi, 2003; d'Adda di Fagagna, 2008) and is characterized by a series of functional hallmarks (Rodier and Campisi, 2011; Lopez-Otin et al., 2013). It is important to note that the DDR remains permanently activated in most senescent cells, as evidenced by the presence of persistent DDF, termed “DNA segment with chromatin alterations reinforcing senescence” (DNA-SCARS; Rodier et al., 2011). These DNA-SCARS, whether telomeric or intra-chromosomal, are suggested DDR activity nodes that maintain long-term DDR signaling (Rodier et al., 2011).

With few exceptions (Coppe et al., 2011), senescent cells from most species and tissues that are triggered by various stresses all display a Senescence-Associated Secretory Phenotype (SASP; **Figure 1B**), which is critical for the ability of these cells to modulate their microenvironment (Coppe et al., 2008, 2010a,b; Ohanna et al., 2011). A large subset of this SASP critically

depends on DDR signaling and is thus an extracellular extension of the DDR (Rodier et al., 2009). The SASP is defined as a pro-inflammatory secretome composed of cytokines (i.e., IL-6 IL-8, GRO α , GRO β , MCP-1), growth factors (i.e., GM-CSF, G-CSF, HGF/SF, IGF), proteases (i.e., metalloproteinase MMP-1, -2, and -3), and other non-soluble extracellular matrix proteins (i.e., collagens, fibronectin, laminin; Bavik et al., 2006; Coppe et al., 2008, 2010a; Ohanna et al., 2011; Malaquin et al., 2013). The exact composition of the SASP, its targets, and the overall downstream outcomes vary considerably depending on the cellular context and the type of stresses, but the consensus is that the SASP is at least partially DDR-dependent and is in major part responsible for modulating senescence-associated inflammatory microenvironments in tissues (Figure 1B).

The SASP contributes to senescence reinforcement in damaged cells and to tissue repair, but also to age-associated tissue dysfunction and other age-related diseases, including cancer (Figure 1B). Because the SASP appears to have both beneficial and deleterious effects, it may represent an interesting, double-edged target for pharmaceutical intervention in human disease (Acosta and Gil, 2012; Perez-Mancera et al., 2014). In the context of cancer, which is particularly applicable to DDR events activated by irradiation or chemotherapy, the SASP also contributes to the clearance of damaged senescent tumor cells by enhancing both innate and adaptive immunity (Xue et al., 2007; Kang et al., 2011; Iannello et al., 2013). However, the SASP also generates chronic inflammation in normal tissues with persistent senescent cells, contributing to age-related tissue dysfunction (Rodier and Campisi, 2011). In the case of

the tumor microenvironment, the SASP of senescent stromal fibroblasts sustains tumor growth and invasion and can even create tumor microenvironments that promote long-term cancer therapy resistance (Krtolica et al., 2001; Sun et al., 2012). Overall, understanding the molecular regulation of the SASP appears essential to reveal how the DDR manages extracellular signaling.

Molecular Regulation of the SASP by the DDR

Direct molecular links between the SASP and the DDR have been demonstrated (Figure 2A), but unlike the rapid extracellular DDR signals, the SASP is a slow, delayed response to DDR signaling. While apical DDR kinases like ATM are activated within minutes of DNA lesions and subsequent DDR transcriptional responses are established within hours by p53 and other transcription factors, the SASP develops over days, with associated factors like IL-6 reaching maximal secretion levels 4–10 days after DDR initiation (Coppe et al., 2008; Rodier et al., 2009). In response to DNA damage, persistent DDR signals emanating from DNA-SCARS are necessary, both for the establishment and maintenance of the SASP (Rodier et al., 2009, 2011). At the molecular level, the DDR proteins H2AX, ATM, NBS1 and CHK2, but not cell cycle arrest mediators p53 and pRb, are required to support the SASP (Rodier et al., 2009, 2011). Activation of the p38MAPK stress kinase pathway also triggers the SASP and in some situations concurrent activation of the

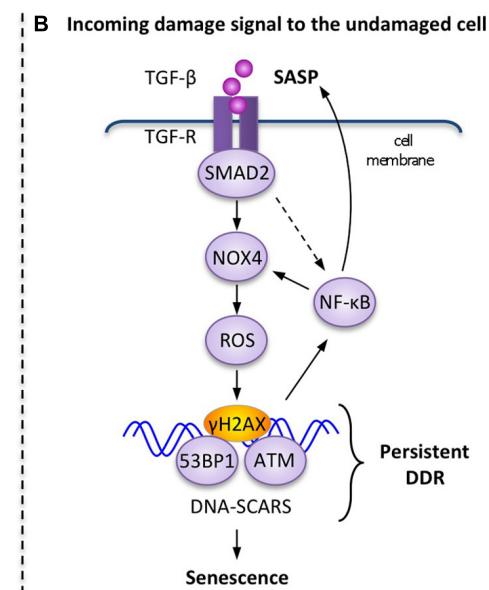
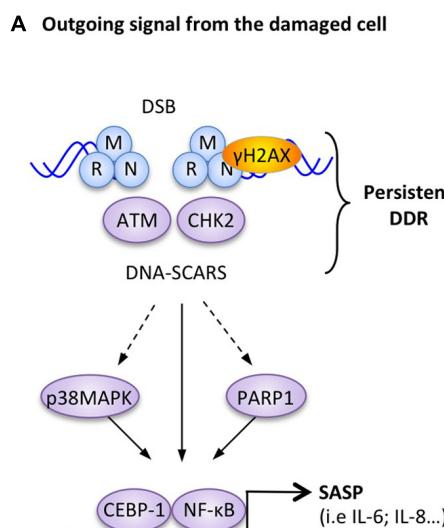


FIGURE 2 | Examples of molecular interactions between the DDR and outgoing-incoming extracellular damage signals. (A) Outgoing signal from the damaged cell: in response to persistent DNA-SCARS, molecular components of the DDR cascade lead to selected transcription factor activation and increased transcription of SASP factors such as IL-6.

(B) Incoming damage to the undamaged cell: the presence of extracellular TGF- β can reinforce DDR-mediated p53 activity and trigger the formation of DNA-SCARS, which subsequently mediate senescence phenotypes, including increased secretion of SASP factors that reinforce a positive senescence feedback loop.

DDR is not necessary suggesting that there may be different subsets of SASP factors requiring varying levels of interaction with the DDR (**Figure 2A**; Freund et al., 2011). For example, the depletion of ATM completely prevents the secretion of IL-6 and IL-8 in senescent irradiated human fibroblasts, but does not impede increased secretion of MCP1, TIMP2, and IGFBP2 (Rodier et al., 2009).

The inflammation-associated transcription factor, nuclear factor- κ B (NF- κ B), is revealing itself to be a master regulator of the SASP (**Figure 2A**; Salminen et al., 2012). The activation of the RelA p65 subunit of NF- κ B and its recruitment to the chromatin are necessary for the expression of several SASP factors, including IL-6 and IL-8 (Chien et al., 2011). Several studies also showed that the DDR can directly trigger activation of NF- κ B signaling via the interaction between activated ATM and the NEMO protein, which is a regulatory subunit of the IKK complex (inhibitor of NF- κ B signaling). DDR activation results in the export of an ATM/NEMO complex into the cytoplasm, where it binds to and activates IKK α/β , leading to the initiation of NF- κ B signaling via the phosphorylation of inhibitory I κ B proteins (Huang et al., 2003; Wu et al., 2006; Miyamoto, 2011). C/EBP β (CCAAT-enhancer-binding proteins), another transcription factor known to be involved in inflammatory regulation, can also contribute to SASP induction in cooperation with NF- κ B (Kuilman et al., 2008).

Alternatively, another important DNA-damage sensor and DDR regulator, known as PARP-1 (Poly-ADP-ribose polymerase 1), is also involved in the regulation of NF- κ B in senescent melanoma cells undergoing the SASP (Ohanna et al., 2011). Perhaps linked in this context, activated PARP-1 can interact with NEMO to enhance the formation of the ATM/NEMO complex (Stilmann et al., 2009).

Cell-Autonomous Reinforcement or Bystander Activation of the DDR Using Late Extracellular DDR Signals (SASP)

Much like the bystander effect described for rapid DDR extracellular signals, the SASP generated from persistently damaged cells is known to modulate DDR-associated behaviors in neighboring cells. Although ROS may influence how the DDR generates the SASP (Guo et al., 2010), most of the SASP's known extracellular effects are currently associated with proteinic soluble factors. Additionally, and again in contrast to the rapid DDR extracellular response, the SASP has been shown to impact both the signal-emitting damaged cell and healthy bystander cells. In damaged cells, the SASP can reinforce p53-associated DDR pathways in a paracrine manner, which maintains senescence in these cells. For example, IL-6 is considered to be a major mediator of paracrine senescence reinforcement (Kuilman et al., 2008). Similarly, CXCR2-binding chemokines (such as IL-8 or GRO-1) are also crucial to reinforce oncogenic- and replication-induced senescence (Acosta et al., 2008). Alternatively, the SASP generated by senescent cells also impacts neighboring bystander cells, as demonstrated both in culture and *in vivo* (Kuilman et al., 2008; Nelson et al., 2012; Acosta et al., 2013). In particular,

multiple SASP components secreted by oncogene-induced senescent cells can trigger paracrine senescence in bystander cells (i.e., TGF β family ligands, VEGF, CCL2, and CCL20) and IL-1 signaling is apparently a major upstream regulator of this paracrine senescence (Acosta et al., 2013). Finally, the SASP factor MCP-1 (CCL2), found in the conditioned media of senescent melanoma cells, was demonstrated to promote DNA lesions in other cells, as illustrated by an increase in 53BP1 DDF (Ohanna et al., 2011). Other extracellular signals that are not necessarily secreted by damaged or senescent cells can also connect to the DDR. For example, type I β -interferon secreted by virally infected cells has been shown to induce paracrine bystander senescence in other cells via the generation of ROS, DDR activation, and p53 activity (Moiseeva et al., 2006).

The link between extracellular signaling and DDR activation is well illustrated by TGF β signaling, which is often associated with senescence (Hubackova et al., 2012; **Figure 2B**). The inhibition of the TGF β pathway resulted in defective DDR activation in irradiated normal cells, as measured by decreased p53 activation and a reduction in ATM, CHK2, and H2AX phosphorylation (Kirshner et al., 2006). The addition of recombinant TGF β -1 also restored functional ATM in damaged normal cells and could induce DDR-associated senescent phenotypes in healthy hepatocellular carcinoma cells (Kirshner et al., 2006; Senturk et al., 2010). Similarly, TGF β -1 from the conditioned media of senescent normal fibroblasts (oncogene-induced senescent, replicative exhaustion, or genotoxic drugs) triggered a senescent growth arrest in undamaged cells via the DDR-associated p53 or the p16 pathways (**Figure 2B**). TGF β -induced bystander senescence is associated with the activation of a persistent DDR, the formation of DNA-SCARS, and the subsequent production of SASP factors. It is probable that the activation of the TGF β /SMAD pathway results in increased intracellular ROS and NOS production in the target bystander cells through an NF- κ B-mediated increase in Nox4 expression and NAPDH oxidase activity (Burdak-Rothkamm et al., 2007, 2008; Shao et al., 2008). Finally, the stimulation of the IL1R/NF- κ B pathway known to activate cellular inflammatory responses also cooperates with TGF β /SMAD to induce bystander senescence (Hubackova et al., 2012).

Conclusion and Perspectives

It is now clear that DNA-damaged cells interact with the extracellular environment to induce bi-directional changes within themselves and in undamaged neighboring cells. These communication strategies have most likely evolved to convey stress signals from damaged cells to the surrounding tissue and occur relatively rapidly (within hours) and/or slowly under the shape of the SASP. In the case of cancer treatment, therapeutic tools, including radiation and cytotoxic drugs, can trigger DDR activity and cellular senescence in normal and neoplastic cells but whether the generation of a DDR-driven immunomodulatory microenvironment has beneficial or detrimental consequences remains unknown (Acosta and Gil, 2012; Sun and Nelson, 2012). It is thus evident

that understanding microenvironment-modulating DDR-related mechanisms and their consequences remains a major challenge in the development of successful cancer therapies. Recent tools have emerged to directly manipulate senescence in mammalian model systems, which will be very useful in determining the importance of extracellular signals emitted from senescent cells (Baker et al., 2011; Laberge et al., 2013; Demaria et al., 2014). The use of these models and other strategies will be instrumental in the exploration of the pathways regulating DDR-mediated extracellular communication, as well as in the identification of extracellular signaling molecules that may become potential targets for therapeutic development in advanced

cancer therapies that take into account tissue microenvironments.

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The DNA damage response in mammalian oocytes

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DNA damage is one of the most common insults that challenge all cells. To cope, an elaborate molecular and cellular response has evolved to sense, respond to and correct the damage. This allows the maintenance of DNA fidelity essential for normal cell viability and the prevention of genomic instability that can lead to tumor formation. In the context of oocytes, the impact of DNA damage is not one of tumor formation but of the maintenance of fertility. Mammalian oocytes are particularly vulnerable to DNA damage because physiologically they may lie dormant in the ovary for many years (>40 in humans) until they receive the stimulus to grow and acquire the competence to become fertilized. The implication of this is that in some organisms, such as humans, oocytes face the danger of cumulative genetic damage for decades. Thus, the ability to detect and repair DNA damage is essential to maintain the supply of oocytes necessary for reproduction. Therefore, failure to confront DNA damage in oocytes could cause serious anomalies in the embryo that may be propagated in the form of mutations to the next generation allowing the appearance of hereditary disease. Despite the potential impact of DNA damage on reproductive capacity and genetic fidelity of embryos, the mechanisms available to the oocyte for monitoring and repairing such insults have remained largely unexplored until recently. Here, we review the different aspects of the response to DNA damage in mammalian oocytes. Specifically, we address the oocyte DNA damage response from embryonic life to adulthood and throughout oocyte development.

Keywords: oocytes, DNA damage response, meiotic recombination, p63, DNA damage checkpoint, meiosis, prophase arrest, apoptosis

THE DNA DAMAGE RESPONSE

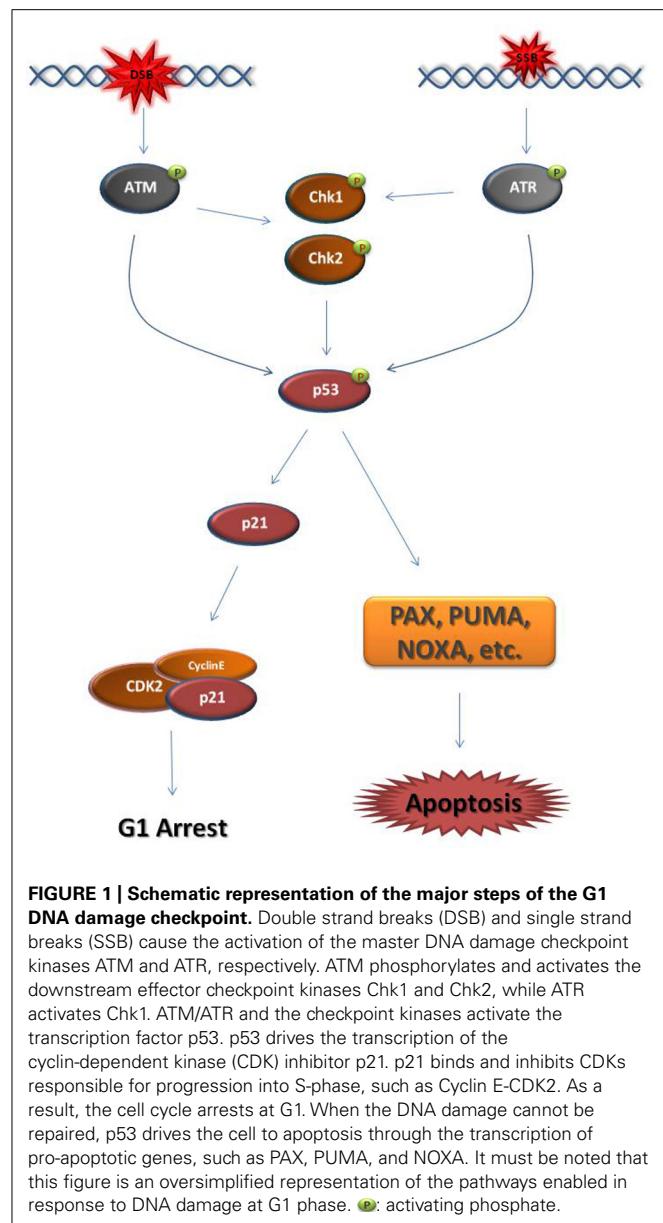
Cells respond to DNA damage created in the form of single strand breaks (SSBs) or double strand breaks (DSBs) by arresting their cell cycle to allow time for the damage to be repaired. Therefore, the DNA damage response (DDR) involves cell cycle arrest through the activation of DNA damage checkpoints (DDCs) and DNA damage repair mechanisms. The DDR sequence of events is tightly coordinated so that cell cycle arrest is lifted as soon as the damage has been repaired. When the extent of damage does not allow full repair, programmed cell death mechanisms become active in order to remove, through apoptosis, the permanently damaged cells (Bartek and Lukas, 2007; Ciccia and Elledge, 2010).

Eukaryotic cells activate DDR mechanisms primarily at the G1/S-phase transition and the G2/M-phase transition. In both cell cycle phases, DSB or SSB establish a DDC by triggering the activation of the master kinases ATM (ataxia telangiectasia mutated) and ATR (ataxia telangiectasia and Rad3-related), respectively (Reinhardt and Yaffe, 2009; Smith et al., 2010). At G1, the major downstream effector of the ATM/ATR kinases is the transcription factor p53, also known as “the guardian of the genome” (Figure 1; Kastan and Lim, 2000; Bartek et al., 2007). When activated, p53 blocks the transcription of cell cycle regulators that normally induce the G1/S-phase transition, such as cyclin E, while driving the transcription of factors that block the G1/S-phase transition, such as the cyclin-dependent kinase (CDK) inhibitor, p21

(Rocha et al., 2003; Mirzayans et al., 2012). p53 is also the primary inducer of apoptotic mechanisms following DNA damage (Fridman and Lowe, 2003; Meulmeester and Jochemsen, 2008).

At G2, establishment of the DDC and subsequent M-phase entry inhibition requires the ATM/ATR-dependent activation of checkpoint kinases, Chk1 and Chk2 (Figure 2; Bartek and Lukas, 2007; Smith et al., 2010). Normally, entry into M-phase is obtained by the activation of the universal M-phase regulator, cyclin B-CDK1 (Doree and Hunt, 2002; Lindqvist et al., 2009). Cyclin B-CDK1 activation requires cyclin B synthesis and the activation of Cdc25 phosphatases which lift CDK1 inhibitory phosphorylations established by CDK1 inhibitors such as Wee1 and Myt1 kinases (Aressy and Ducommun, 2008; Potapova et al., 2009). Following DNA damage at G2, Chk1/Chk2 kinases cause the inhibition of cyclin B-CDK1 activation by disrupting the action of Cdc25 either through facilitating SCF (Skp, Cullin, F-box) ligase-dependent degradation, as in the case of Cdc25A or through inhibitory phosphorylation (Cdc25B, Cdc25C; Mailand et al., 2000; Busino et al., 2003; Ferguson et al., 2005; Boutros et al., 2007).

During the DDC-mediated arrest, DNA damage is repaired by a number of different mechanisms depending on the nature of the damage. Single strand damage is repaired by three main repair pathways: base excision repair (BER), nucleotide excision repair (NER), and mismatch repair (MMR). Two are the major mechanisms involved in DSB repair, namely homologous recombination

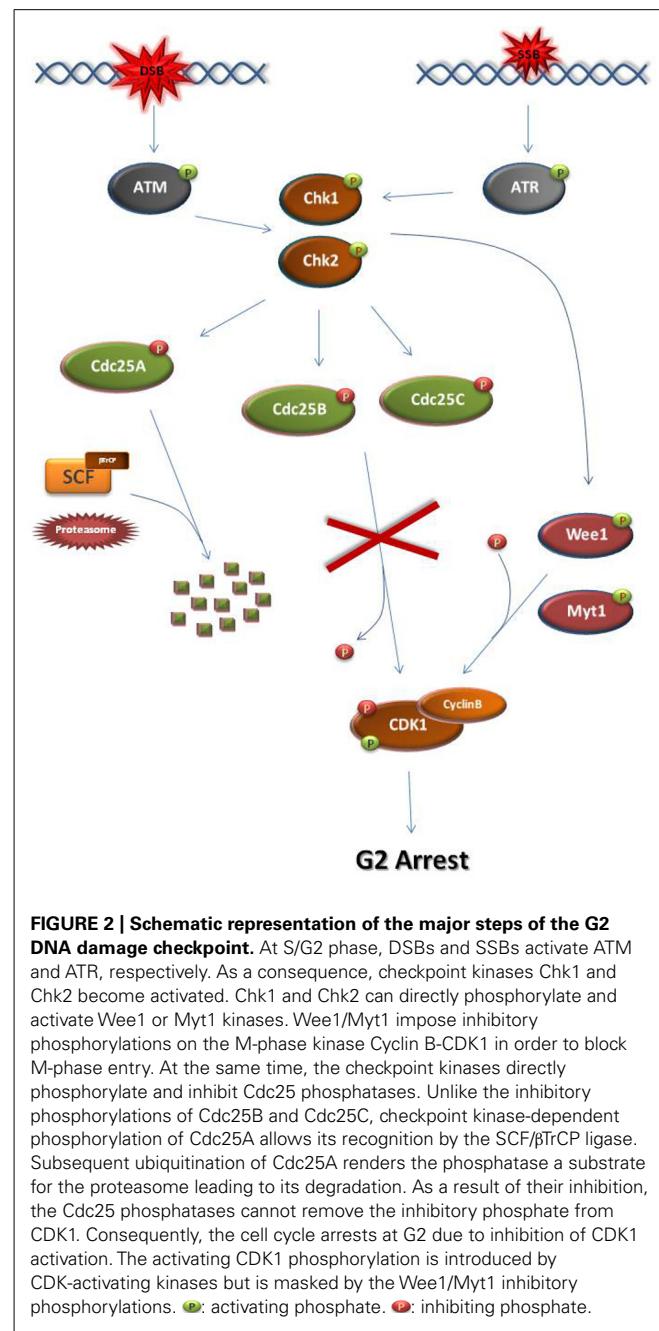


(HR) and non-homologous end joining (NHEJ; Aguilera and Gomez-Gonzalez, 2008; Cohn and D'Andrea, 2008).

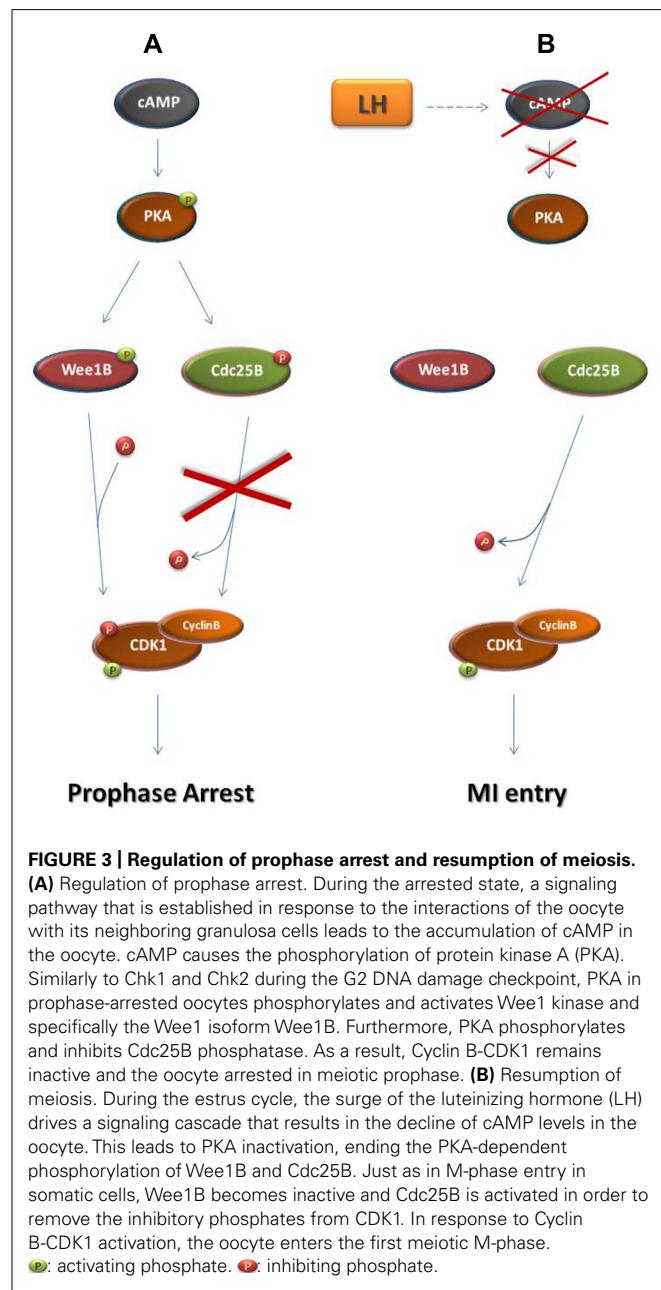
PROPHASE ARREST

The oocyte is a unique cell that differs significantly both from somatic cells but also from the male germ cells in respect to its cell cycle, its functions and its purpose. A unique characteristic of the oocyte, not seen in any other cell type, is prophase arrest.

The mechanisms regulating meiotic prophase arrest and resumption of meiosis resemble the establishment of the somatic cell G2 DDC and checkpoint recovery, respectively (Figures 2 and 3; Bassermann et al., 2008; Solc et al., 2010). The major common element in both systems is the alteration of cyclin B-CDK1 activity, predominantly through the action of CDK1 activators and inhibitors (Bassermann et al., 2008; Solc et al., 2010).



Before the end of gestation oocytes become arrested at the dictyate stage of the meiotic prophase (Rodrigues et al., 2008). During prophase arrest in oocytes, cyclin B-CDK1 remains inactive due to the maintenance of high levels of cAMP within the oocyte and the subsequent sustained activation of protein kinase A (PKA; Figure 3; Mehlmann et al., 2002; Schmitt and Nebreda, 2002). PKA phosphorylates and inactivates the Cdc25 isoform Cdc25B which is responsible for cyclin B-CDK1 activation in oocytes (Lincoln et al., 2002; Pirino et al., 2009; Oh et al., 2010). Furthermore, PKA phosphorylates and activates the CDK1 inhibitor Wee1B which is the oocyte-specific Wee1 isoform (Han et al., 2005; Oh et al., 2010). Following the rise in the levels of luteinizing hormone



(LH), during the estrus cycle, cAMP levels drop and PKA becomes inactive allowing CDC25B activation and the subsequent cyclin B-CDK1 activation leading to entry into the first meiotic M-phase (MI; Lincoln et al., 2002; Marangos and Carroll, 2004; Solc et al., 2010).

Most of the information we possess regarding the mammalian oocyte DDR involves prophase arrest.

MEIOTIC RECOMBINATION CHECKPOINTS

In mammalian oocytes, DNA breaks are first identified during meiotic recombination. Meiotic recombination is a process that takes place before birth from the leptotene to the pachytene stage of meiotic prophase and involves the natural formation of DSBs.

Since meiotic recombination processes are extensively reviewed elsewhere (Lydall et al., 1996; Roeder and Bailis, 2000; Burgoyne et al., 2009; Martinez-Perez and Colaiacovo, 2009; Kurahashi et al., 2012) we will limit our analysis to a very general overview of the major aspects of the recombination-induced DDR in mammalian oocytes.

At the start of meiotic prophase (embryonic day 13–18.5 post-coitus in female mice), homologous chromosomes pair along their full length in a process called synapsis (Roeder, 1997; Llera et al., 2008; Martinez-Perez and Colaiacovo, 2009). During synapsis and following the initiation of homolog pairing, DNA DSBs appear within the chromosomes. These DSBs allow DNA exchange between homologous non-sister chromatids through genetic recombination (McDougall et al., 2005; Burgoyne et al., 2009). Recombination leads to the formation of natural bridges, called chiasmata, which hold the homologous chromosomes together until MI allowing their attachment from the opposite poles of the MI spindle and their alignment at the metaphase I plate (McDougall et al., 2005; Burgoyne et al., 2009). Therefore, formation of chiasmata during meiotic recombination ensures the correct segregation of homologous chromosomes during the first meiotic division. Meiotic recombination dysfunctions can cause damaged genomes and the formation of aneuploid gametes (Burgoyne et al., 2009; Yanowitz, 2010; Kurahashi et al., 2012). Therefore, meiotic cells have developed checkpoint mechanisms around the pachytene stage of meiotic prophase in order to ensure the integrity and the completion of recombination (Lydall et al., 1996; Roeder and Bailis, 2000). In mammals, the activation of the recombination pachytene checkpoint when meiotic cells do not complete HR in time leads to cell death through apoptosis (Lydall et al., 1996; Roeder and Bailis, 2000). In oocytes, dysfunction of factors involved in the recombination process leads to apoptosis at the perinatal period (Pittman et al., 1998; Baudat et al., 2000; Di et al., 2005).

In meiotic recombination, it is well established that Spo11 is the main factor to promote the formation of DSBs (Baudat et al., 2000; Di et al., 2005). However, recent findings have shown that homolog pairing is completely abolished in Spo11^{-/-} spermatocytes suggesting that Spo11 is also required for the DSB-independent initiation of synapsis (Boateng et al., 2013). Absence of the Spo11-dependent homolog pairing and DSB formation leads to oocyte apoptosis during early follicular development, soon after birth (Baudat et al., 2000; Di et al., 2005). Similar observations are made in mice lacking Spo11-associated proteins, such as Mei4 (Kumar et al., 2010). In Spo11 null mice, the oocytes that survive and acquire the competence to enter M-phase cannot segregate their homologous chromosomes properly due to the absence of chiasmata and remain arrested at MI (Cole et al., 2010).

Other factors, such as ATM and DMC1 are responsible for rejoicing the DNA strands (Pittman et al., 1998; Yoshida et al., 1998; Roeder and Bailis, 2000; Di et al., 2005). Besides its role in DDC establishment, ATM is a crucial component of HR repair mechanisms (Smith et al., 2010). The importance of these proteins in DNA strand rejoicing is shown by the fact that the absence of DMC1 or ATM leads to programmed cell death in prophase oocytes and DMC1 null and ATM null mice are infertile as are Spo11 null mice (Pittman et al., 1998; Yoshida et al., 1998; Baudat et al.,

2000; Di et al., 2005). However, in the case of DMC1 and ATM, the oocytes do not reach the stage of becoming enclosed in follicles and degenerate, through apoptosis earlier than Spo11 null oocytes (Pittman et al., 1998; Yoshida et al., 1998; Baudat et al., 2000; Di et al., 2005). Furthermore, in DMC1-Spo11 and ATM-Spo11 double mutants the oocyte reserve depletion phenotype resembles the one seen in Spo11 null mice, which leads to the conclusion that the Spo11 mutation is epistatic to the DMC1 and ATM mutations (Di et al., 2005). These results indicate that, unlike Spo11 mutants, the different phenotype of the DMC1 and ATM mutants is possibly the result of persistent, unrepaired DNA damage.

Besides ATM, other traditional ATM-dependent DDR factors are activated at the sites of meiotic recombination-induced DNA damage in order to amplify the DSB signal, such as ATR kinase, BRCA1 and the phosphorylated form of the nucleosomal histone H2AX (γ H2AX; Xu et al., 2003; Burgoyne et al., 2007). However, in the absence of DSBs, ATR, BRCA1, and γ H2AX are recruited on unsynapsed homologous chromosomes in order to impose their transcriptional silencing (Turner et al., 2005; Mahadevaiah et al., 2008; Burgoyne et al., 2009). If synapsis is not successful, transcriptional silencing can lead to apoptosis if important active genes cease to function (Burgoyne et al., 2009; Kurahashi et al., 2012). This DSB-independent process allows the elimination of oocytes with unsynapsed chromosomes and could explain the Spo11 $^{-/-}$ oocyte death phenotype.

Therefore, there appear to be two checkpoint responses to recombination defects in oocytes: a DNA DSB-dependent response triggered by unrepaired DSBs and a DNA DSB-independent response triggered by the absence of synapsis. In both cases, the activation of the checkpoint will lead to apoptosis. However, it is not yet determined how unrepaired, recombination-induced DSBs would trigger apoptosis in oocytes.

p63-DEPENDENT PATHWAY

In mammalian oocytes, DSBs induced as a consequence of genotoxic stress trigger the activation of a TA δ p63-dependent mechanism which drives affected oocytes to apoptosis (Suh et al., 2006; Kerr et al., 2012a).

TA δ p63 is an isoform of p63 which belongs to the p53 family of transcription factors. This protein family includes three transcription factors, namely p53, p63, and p73 (Levine et al., 2011). Besides being important for the activation of DDR mechanisms, mainly cell cycle arrest and apoptosis of damaged cells, these factors also possess a wide range of other functions including their involvement in maternal reproductive efficiency. p53 has been shown to regulate embryo implantation (Hu et al., 2007). TA δ p73, a p73 isoform, is involved in the M-phase spindle assembly checkpoint and mice lacking TA δ p73 are infertile. In female TA δ p73 $^{-/-}$ mice, infertility is due to chromosome missegregation leading to chromosomal abnormalities in the dividing oocyte and pre-implantation stage embryo (Tomasini et al., 2008; Levine et al., 2011). TA δ p63, a p63 isoform, is the only p53 family member identified so far to participate in the oocyte DDR. Although, TA δ p63 is not expressed in the male germ cells, a newly identified hominidae isoform, GTAp63, seems to possess DDR functions in males (Beyer et al., 2011; Amelio et al., 2012).

TA δ p63 is found in the nucleus of oocytes enclosed in primordial, primary and early pre-antral follicles (Figure 4) but is completely lost in the more mature, antral, follicles (Suh et al., 2006). TA δ p63 expression begins at embryonic day 18.5 up to adulthood (Suh et al., 2006; Livera et al., 2008). p63 has also been found in human embryonic stage oocytes (Livera et al., 2008). Nevertheless, TA δ p63 seems to be completely dispensable for oogenesis and the loss of TA δ p63 does not affect the oocyte reserve. The importance of TA δ p63 for the oocyte DDR was first identified in TA δ p63 null mice. In wild type and p53 $^{-/-}$ animals, ionizing radiation causes the complete deterioration and loss of primordial follicles, while the larger pre-antral follicles remain unaffected. However, the oocytes in primordial follicles of the TA δ p63 null mice were resistant to irradiation and cell death (Suh et al., 2006). These experiments showed that TA δ p63 induces cell death in primordial follicle oocytes with damaged DNA and that this function is not shared with p53. It must be noted that p63 seems to be only involved in DNA damage-dependent apoptosis since the rate of physiological embryonic oocyte death in p63 $^{-/-}$ ovaries is not different from wild type ovaries (Livera et al., 2008).

It has been proposed that, following DNA damage in oocytes, TA δ p63 becomes activated through phosphorylation by c-Abl tyrosine kinase (Gonfloni et al., 2009). Gonfloni and colleagues have shown that c-Abl inhibition by imatinib or GNF-2 protected oocytes from apoptosis in response to cisplatin-induced DNA damage (Gonfloni et al., 2009; Maiani et al., 2012). p63 phosphorylation drives resting inactive dimmers to form tetramers which possess the ability to bind DNA and activate the transcription machinery (Deutsch et al., 2011). The possible mechanism for Tap63 activation could involve the DNA damage-induced activation of the stress kinase c-Jun N-terminal kinase (JNK). In somatic cells, JNK phosphorylates the 14-3-3 proteins which under physiological conditions sequester c-Abl in the cytoplasm (Yoshida et al., 2005). 14-3-3 phosphorylation releases c-Abl to become transported into the nucleus in order to phosphorylate and activate Tap63. However, there has been some skepticism regarding c-Abl involvement because pharmacological agents, such as imatinib have occasionally been unable to inhibit oocyte apoptosis (Kerr et al., 2012b; Maiani et al., 2012). At the moment, it appears that the controversy surrounding c-Abl would only be resolved conclusively by genetically removing c-Abl from the female germ line.

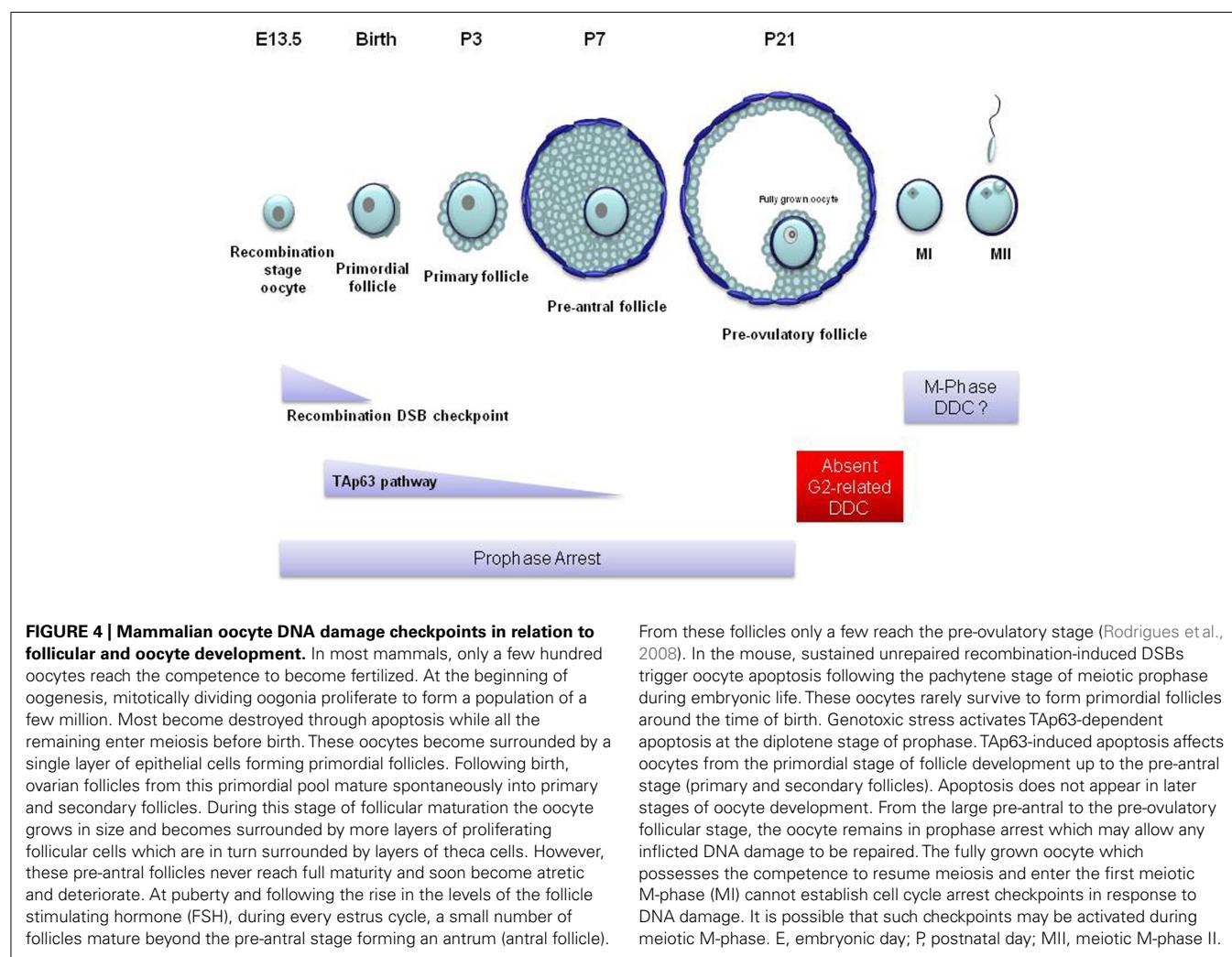
Another very important question, however, is: which are the transcriptional targets of Tap63 that trigger apoptosis? Recently, two such targets have been identified in mouse oocytes, namely PUMA and NOXA (Kerr et al., 2012c). Both proteins belong to the pro-apoptotic arm of the Bcl-2 family and they have been known to inhibit pro-survival Bcl-2 proteins and promote the function of BAX and BAK, two major pro-apoptotic Bcl-2 family members, which in turn enable the mitochondria-induced apoptosis mechanisms (Chipuk and Green, 2008; Youle and Strasser, 2008). TA δ p63 enables the transcription of both PUMA and NOXA in mouse primordial follicle oocytes. In addition, PUMA $^{-/-}$ mice and especially the double mutants PUMA $^{-/-}$ NOXA $^{-/-}$ mice, do not lose their primordial follicle pool in response to genotoxic stress (Kerr et al., 2012c). Therefore, TA δ p63-dependent PUMA and

NOXA expression is most possibly responsible for driving oocyte apoptosis following DNA damage.

The knock-out mouse models of TAp63, PUMA and NOXA have shown that inhibition of the TAp63 pathway can rescue the primordial follicle oocyte pool from apoptosis following DNA damage. These observations could open novel medical options in order to sustain the fertility of women undergoing cancer therapy. It is well known that chemotherapy and radiation therapy for treating cancer leads to depletion of the ovarian oocyte reserve and leads to premature ovarian failure (POF) and hence premature menopause (Maltaris et al., 2007). Therefore, possible treatments that are based on the inhibition of the TAp63 pathway could allow the preservation of the oocyte pool following cancer therapy. However, is it safe to allow damaged oocytes to survive following cancer treatment? It would be expected that these oocytes carry significant damage that could be transferred to their offspring. However, an exciting result refutes these concerns: although wild type mice lose their primordial follicle reserve and become infertile following genotoxic stress, PUMA^{-/-} and PUMA^{-/-} NOXA^{-/-} female mice exposed to ionizing radiation have viable, healthy, and fertile offspring at the same rate as wild type mice not exposed to DNA

damage (Kerr et al., 2012c). This finding suggests that during their long prophase arrest, oocytes possess the ability to repair DNA damage efficiently. Although more work needs to be done before treatments are obtained, these observations bring hope to cancer patients facing infertility.

The fact that genotoxic stress does not prevent the preservation of healthy oocytes when the TAp63 pathway is inhibited, raises an interesting question: why is a TAp63-dependent apoptotic pathway needed in oocytes? The answer might lie slightly before TAp63 expression, at the time of meiotic recombination. Quite conveniently, TAp63 is expressed following the physiological recombination-induced DSB repair. Immunofluorescence experiments show that γH2AX foci representing recombination-induced DSBs do not co-exist with TAp63. In mouse oocytes, γH2AX staining disappears by E18.5 by which time TAp63 becomes apparent (Livera et al., 2008). In this way, oocytes undergoing meiotic recombination are not in danger of apoptosis. However, as previously discussed, sustained DSBs during recombination, trigger the establishment of oocyte death mechanisms. The activation of these processes during and following the pachytene stage of prophase coincides with the appearance of TAp63. Therefore, TAp63 may



be the “guardian of meiotic recombination,” driving to apoptosis any oocytes that fail to rejoin their chromosome arms on time.

These observations support the hypothesis that the original role, in evolutionary terms, of TAp63 may be the protection of the gene pool from meiotic recombination failure and not necessarily from externally inflicted genotoxic insults. Therefore, the deleterious role of TAp63 following exogenous genotoxic stress might be an undesired remnant of a p63-related recombination checkpoint.

At the moment, it remains unknown what processes take place at the late pre-antral follicular stage that would lead to the disappearance of TAp63 and TAp63-dependent apoptosis. Nevertheless, it is a fact that a p63-dependent apoptotic mechanism is absent in antral follicles. Therefore, an important question that arises is how the non-apoptotic mature antral and pre-ovulatory follicles (**Figure 4**) respond to oocyte DNA damage.

PROPHASE TO MI TRANSITION

From the antral and up to the pre-ovulatory follicle, the response to DNA damage may involve the activation of repair mechanisms alone. At these stages of follicular development, physiological prophase arrest means that a DNA damage-induced checkpoint is not required to halt the cell cycle in order to permit repair. However, the fully grown oocyte in the pre-ovulatory follicle would not be expected to respond to DNA damage solely by repair mechanisms, but also by cell cycle arrest checkpoints. At this stage, the oocyte has reached full cytoplasmic and nuclear development and has acquired the competence to enter meiotic M-phase as soon as the LH surge occurs (Eppig, 1996). In the mouse, cell cycle regulators that are important for M-phase entry, such as cyclin B, CDK1, and Cdc25 accumulate in the fully grown, pre-ovulatory oocyte (Kanatsu-Shinohara et al., 2000).

Considering the resemblance of meiotic prophase arrest and the G2 DDC, it would be anticipated that fully grown oocytes employ similar DDR mechanisms as the ones present in somatic cell G2 phase. Therefore, it is surprising that a DDC is not established in response to DNA damage in M-phase competent oocytes.

Studies in mouse oocytes have shown that radiation-induced DNA damage may cause chromosomal aberrations, such as aneuploidy, translocations, chromatid interchanges and breaks (Tease, 1983; Jacquet et al., 2005). Past studies hinted at the possibility of a limited DDR in fully grown oocytes (Mailhes et al., 1994; Bradshaw et al., 1995). More specifically, it has been shown that a significant delay in the duration of MI is not observed following injection into female mice of etoposide, a topoisomerase II inhibitor and DSB inducer (Mailhes et al., 1994; Bradshaw et al., 1995).

Recently, the fully grown oocyte DDR has been examined in greater detail. In fully grown oocytes, DNA damage in the form of DSBs, that would normally cause G2 arrest in somatic cells, does not affect the timing and rate of entry into M-phase (Marangos and Carroll, 2012). Although, a DDC is not being established efficiently, DNA damage detection is effective. This has been determined by the presence of γ H2AX at the DSB sites. A DDC is only established following very severe DNA damage inflicted by high concentrations of Etoposide or the DNA intercalating agent Doxorubicin, causing a significant delay in M-phase entry (Marangos and Carroll, 2012). Similar observations were

also seen with the use of another DSB-inducing agent Neocarzinostatin (Yuen et al., 2012). Nevertheless, even following severe DNA damage and prolonged arrest, oocytes will eventually enter M-phase. The failure to establish a DDC in prophase-arrested oocytes could be attributed to checkpoint adaptation: a mechanism, which in somatic cells, involves Polo-like kinase 1 (Plk1) and Claspin and leads to the eventual inactivation of the G2 DDC in the presence of irreversible DNA damage (Yoo et al., 2004; Syljuasen et al., 2006).

The molecular basis for the absence of a reliable DDC in response to DSBs appears to be due to a limited ability to activate ATM kinase (Marangos and Carroll, 2012). The lack of ATM activity also affects the activation levels of downstream effectors such as Chk1. Low levels of expression of ATM in fully grown oocytes could be the reason for limited ATM activity. Another possibility could be the distinct chromatin configuration in fully grown oocytes (Marangos and Carroll, 2012). The fully grown oocyte is subjected to chromatin histone modifications such as deacetylation and methylation which are crucial for chromatin condensation and transcriptionally inactive heterochromatin formation (Mattson and Albertini, 1990; De La Fuente, 2006; Ma et al., 2012). Considering that the DDR and ATM specifically are known to be influenced by changes in chromatin structure and chromatin condensation (Bakkenist and Kastan, 2003), one hypothesis might be that DDR mechanisms are either not able to engage or are not triggered due to the fully grown oocyte specialized chromatin configuration.

The induction of Cdc25A degradation and Cdc25B inactivation are also inhibited following DNA damage in fully grown oocytes. The lack of Cdc25A destruction appears to be independent of ATM activity on account of the fact that Cdc25A is still present following high levels of DNA damage when ATM and Chk1 are active. However, the inability of DSBs to block Cdc25B activity seems to be ATM/Chk1-dependent since high levels of DNA damage cause a dramatic inhibitory phosphorylation of the phosphatase (Marangos and Carroll, 2012). Cdc25B inactivation could explain the sustained prophase arrest observed following significant levels of damage. This is not surprising considering that Cdc25B is irreplaceable in oocytes and the absence of Cdc25B, as in Cdc25B null mice, leads to female infertility due to the inability of oocytes to enter M-phase (Ferguson et al., 2005).

Besides DSBs, another type of highly toxic DNA lesions, inter-strand crosslinks (ICLs), do not appear to activate an efficient DDR. In fully grown mouse oocytes, a major ICL repair factor, the Fanconi Anemia protein FANCD2 fails to be recruited to the sites of the DNA lesions (Yuen et al., 2012). Therefore, ICLs are not being repaired. Nevertheless, the oocytes enter M-phase without any delay. An explanation for these observations could be the possible absence of the activity of the ATM-related kinase, ATR. In somatic cells, ATR and its downstream effector Chk1 become active and enable a checkpoint in response to ICLs (Wang, 2007; Ben-Yehoyada et al., 2009). ATR is also required for the efficient monoubiquitination of FANCD2 enabling its role as an ICL repair factor (Andreassen et al., 2004). It would be interesting to see whether ATR can become active in fully grown oocytes in response to DNA damage. It is possible that, as in the case of ATM, ATR is either not expressed or unable to become recruited to the oocyte

chromatin. This could explain the absence of FANCD2 and the subsequent inefficiency of the Fanconi Anemia pathway in fully grown oocytes.

It is not yet clear why fully grown oocytes cannot activate major DDR factors, such as ATM or repair factors, such as FANCD2. Although we have provided some possible explanations, further work is necessary in order to understand the mechanisms involved. Irrespective of how the system functions, it may be that oocytes have the capacity to resolve DNA lesions later in the cell cycle, perhaps during MI or MII, or even after fertilization during early embryonic development.

MEIOTIC M-PHASE RESPONSE TO DNA DAMAGE

When a follicle reaches the pre-ovulatory stage it responds to the surge of LH and as a result the fully grown oocyte exits prophase arrest and enters MI. Resumption of meiosis leads to the first meiotic division and the extrusion of the first polar body (Pb1) which contains half of the homologous chromosomes and a minimum amount of cytoplasm. The oocyte then enters the second M-phase (MII) without an intervening interphase. It is at this stage the oocyte is ovulated and fertilization takes place. Egg activation triggers the completion of the second meiotic division and entry into the first embryonic cell cycle.

Considering the inability of meiotic prophase to establish a DDC, the two meiotic M-phases pose the only possible line of defense against DNA damage inflicted to the fully grown oocyte before the damage reaches the developing embryo. However, the knowledge on possible meiotic M-phase DDR mechanisms is extremely limited. When fully grown oocytes are exposed to the DSB-inducing agent Neocarzinostatin MI division is blocked (Yuen et al., 2012). It is not yet known, however, how sensitive this

M-phase arrest is and which factors are implicated. Interestingly, the presence of ICLs in either MI or MII does not inhibit or delay cell division. However, ICLs formed in oocytes affect the quality and development of the resulting pre-implantation embryos (Yuen et al., 2012). Therefore, it seems that, the decision to establish or not an M-phase DDC depends on the type of DNA damage. More work needs to be done, in order to clarify the M-phase response to DNA damage.

CONCLUDING REMARKS

The experimental evidence of especially the last decade has shed light into the diverse ways by which the mammalian oocyte responds to DNA damage. From our current knowledge we can assume that a DDC is necessary, primarily, for avoiding meiotic recombination errors in order to ensure correct chromosome segregation during the meiotic divisions. After birth, the TAp63-dependent checkpoint appears to be dispensable. The absence of apoptosis in damaged primordial follicle oocytes is not detrimental probably because the oocytes remain arrested at prophase where they have the time to repair any inflicted DNA damage. However, at the moment when a DDC is mostly needed, when the oocyte acquires the competence to enter M-phase, cell cycle arrest mechanisms that would respond to DNA damage are absent. It seems that the oocytes find preferable for DNA damage to be confronted later, in M-phase or the early embryonic cell cycles. Nonetheless, many important questions are still unanswered: is the recombination DDC a p63-dependent apoptosis mechanism? Why does the fully grown oocyte choose not to activate DDCs? What mechanisms are recruited in meiotic M-phase to respond to DNA damage? Therefore, there are still many pieces to be found in the puzzle that is the DDR of mammalian oocytes.

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ARF: a versatile DNA damage response ally at the crossroads of development and tumorigenesis

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Alternative reading frame (ARF) is a tumor suppressor protein that senses oncogenic and other stressogenic signals. It can trigger p53-dependent and -independent responses with cell cycle arrest and apoptosis induction being the most prominent ones. Other ARF activities, particularly p53-independent ones, that could help in understanding cancer development and provide potential therapeutic exploitation are underrated. Although ARF is generally not expressed in normal tissues, it is essential for ocular and male germ cells development. The underlying mechanism(s) in these processes, while not clearly defined, point toward a functional link between ARF, DNA damage and angiogenesis. Based on a recent study from our group demonstrating a functional interplay between ataxia-telangiectasia mutated (ATM) and ARF during carcinogenesis, we discuss the role of ARF at the crossroads of cancer and developmental processes.

Keywords: ARF, ATM, angiogenesis, ocular development, vascular network, involution, meiosis, spermatogenesis

INTRODUCTION

The ARF (p14^{ARF} in humans, p19^{ARF} in mice) tumor suppressor is encoded by the INK4A/ARF locus that also harbors another onco-suppressor, namely the cyclin-dependent kinase inhibitor p16^{INK4A} (Quelle et al., 1995; Sherr, 2006). The p16^{INK4A} protein maintains pRB in an active form to inhibit E2F activity (Tsantoulis and Gorgoulis, 2005; Sherr, 2006). In this way S-phase entry and therefore cell division is prevented (Sherr, 2006). On the other hand, ARF is a “sensor” of various stresses including oncogenic ones, like aberrant expression of Myc, E1A and RAS (de Stanchina et al., 1998; Zindy et al., 1998; Palmero et al., 1999). Other stresses that can also activate ARF are oxidative stress and heat shock (Damalas et al., 2011; Liontos et al., 2012). In response it can act both in p53-dependent and -independent manners (Weber et al., 2000; Kotsinas et al., 2014), triggering either growth arrest or apoptosis to counteract abnormal cell proliferation (Sherr, 2006). Apart from cancer (Sherr, 2006), accumulating data highlight ARF as a versatile protein implicated in various physiological processes including developmental ones

(Thornton et al., 2005; Gromley et al., 2009; Churchman et al., 2011), immunomodulation (Través et al., 2012) and ribosomal ribonucleic acid (rRNA) maturation (Sugimoto et al., 2003), as well as pathological ones, such as atherogenesis (González-Navarro et al., 2010). Most of the best known ARF functions are p53-dependent ones (Sherr, 2006), while independent activities seem to be underrated.

Deficiency of ARF or p53, has revealed different phenotypes in mice. Specifically, ARF-null animals mainly develop sarcomas, whereas p53-null animals are predominantly characterized by the evolution of lymphomas (Kamijo et al., 1999). This finding was among the first experimental indications that ARF and p53 can signal independently of each other and not necessarily in a strict linear signaling pathway. Therefore, they may fulfill different tasks in tumor surveillance. Moreover, recent evidence from our group has highlighted the functional significance of a cross-talk among ARF and ATM (Velimezi et al., 2013; Kotsinas et al., 2014) and how ARF can act as an “auxiliary” tumor suppressive mechanism throughout cancer progression in case the DDR pathway is compromised (Velimezi et al., 2013).

In various normal tissues ARF is not expressed. Striking exceptions are the developing oculus (eye), testicular tissue and umbilical arteries (Thornton et al., 2005; Freeman-Anderson et al., 2009; Gromley et al., 2009; Churchman et al., 2011). The underlying mechanism(s) taking place in these tissues, while not clearly defined, point toward a functional link between ARF, DNA damage and angiogenesis.

Abbreviations: ARF, alternative reading frame; ATM, ataxia-telangiectasia mutated; DDR, DNA damage response; DMSO, dimethyl sulfoxide; DSBs, double-strand breaks; FSH, follicle-stimulating hormone; HA, hyaloid artery; HIF1α, hypoxia-inducible factor 1α; HR, homologous recombination; HVS, hyaloid vascular system; IRES, internal ribosome entry sequence; LH, luteinizing hormone; MEFs, mouse embryonic fibroblasts; MVD, microvessel density; NPM/B23, nucleophosmin; NSCLC, non-small cell lung cancer; P, postnatal day; PDGFβ, platelet-derived growth factor β; PDGFRβ, platelet-derived growth factor receptor β; PHPV, persistent hyperplastic primary vitreous; pRb, retinoblastoma protein; sh, short-hairpin; VEGF, vascular endothelial growth factor; YFP, yellow fluorescent protein.

Considering the ATM and ARF interplay in carcinogenesis (Velimezi et al., 2013), we discuss in this article the role of ARF at the crossroads of cancer and developmental processes. We present the current knowledge regarding the role of ARF in development, particularly during spermatogenesis and ocular development in mice. Furthermore, we provide data (including unpublished ones) consolidating the notion that the interference with vascular dynamics accounts for a novel, inherent p53-independent tumor suppressive property of ARF that could be therapeutically exploited in p53-deficient tumors.

THE ROLE OF ARF IN MALE GERM CELL DEVELOPMENT: A MATTER OF PRESERVING GENOMIC INTEGRITY

Spermatogenesis is a spatio-temporally coordinated process by which undifferentiated spermatogonia (i.e., the stem cell population of germinal cells residing on the basement membrane of semiferous tubules) evolve into spermatocytes in the lumen through a series of mitotic and meiotic cellular divisions (Cooke and Saunders, 2002). In mice, Gromley et al. (2009) reported that in this developmental process ARF is selectively expressed in mitotic spermatogonia, but not in intratubular spermatocytes that stain positive for meiotic markers (Figure 1). Intriguingly, in the absence of ARF the testicles of mice exhibit atrophy and produce much lower quantity of sperm compared to wild-type animals. These phenotypic changes are accompanied by increased levels of apoptosis in germ cells during all their developmental stages. Of note, abolition of this transient expression of ARF during spermatogenesis is sufficient to compromise this developmental process throughout the whole reproductive life of mice (Gromley et al., 2009).

Subsequent experiments carried out by the same research group demonstrated that ARF is essential for normal meiotic progression and survival of spermatocytes via initiation of a feed-forward program in their progenitors, the spermatogonia. Interestingly, ARF expression in spermatogonia did not exert an anti-proliferative effect, as they also expressed cyclin D1. Testicular atrophy and reduced production of mature sperm in ARF-deficient mice was not mechanistically related to a disturbed pituitary-gonadal axis and deregulated levels of circulating FSH or LH. Rather, in the absence of ARF a marked increase in the number of spermatocytes undergoing p53-dependent apoptosis at the stage of pachytene of prophase I was observed (Churchman et al., 2011).

Of note, during HR in meiosis the topoisomerase-II like Spo11 enzyme normally causes DSBs, which in turn trigger the activation of ATM and the generation of γ -H2AX foci selectively at the leptotene and zygotene stages (Inagaki et al., 2010). At the pachytene stage when synapsis of homologous chromosomes has been completed, γ -H2AX foci are normally not detected. However, *in situ* analyses showed that in an ARF deficient background, the number of γ -H2AX foci in pachytene spermatocytes is significantly increased (Churchman et al., 2011). The latter observation was therefore suggestive of meiotic defects that have deleterious effects on spermatocytic genomic integrity. Additional evidence for meiotic abnormalities included the identification of asynaptic regions as well as decreased number of foci of the Rad51 and Dmc1 recombinases known to be associated with the repair of DSBs that occur during HR. Overall, the authors concluded that ARF through a

yet poorly understood mechanism, interferes with HR to preserve the fidelity of meiosis in spermatocytes and to protect them from DNA damage and p53-dependent apoptosis (Churchman et al., 2011). Whether the apoptotic death of spermatocytes is a consequence of DNA damage *per se* or not, remains to be elucidated. Inhibition rather than induction of p53-dependent apoptosis by ARF is a unique feature of male germ cells and reveals an opposite to the well-established p53-mediated pro-apoptotic role of ARF in somatic cells (Lowe and Sherr, 2003).

Importantly, ARF is not the only DNA damage-related protein that interferes with the spermatogonial program (Figure 1). The DDR kinase ATM is actually essential for the maintenance of undifferentiated spermatogonia and for retaining their stemness (Barlow et al., 1998; Takubo et al., 2008). In murine testicles, ATM deficiency progressively results in the depletion of undifferentiated spermatogonia. This is functionally associated with cell cycle arrest, loss of genomic integrity and defects at the pre-meiotic level (Barlow et al., 1996, 1998; Elson et al., 1996; Xu et al., 1996; Takubo et al., 2008). Mechanistically, the absence of ATM is linked to the accumulation of DNA damage and the activation of an ARF/p53/p21^{WAF1/Cip1} – dependent growth restrictive pathway. Notably, in transplantation assays where spermatogonia are delivered into the seminiferous tubules of mutant mouse strains that exhibit defective spermatogenesis, p21^{WAF1/Cip1} deficiency is able to restore spermatogonial repopulation ability in an ATM-null background (Takubo et al., 2008).

Taken together, all the above data pinpoint that both ARF and ATM are critical factors for the maintenance of spermatogonia and survival of their progeny during male germ cell development (Figure 1; Barlow et al., 1996; Takubo et al., 2008; Churchman et al., 2011). This common feature parallels with their same function as tumor suppressors in somatic cells. Nevertheless, the imposed outcomes are different in each cell type, suggesting a functional bimodality. In somatic cells ATM and ARF induce cell growth restrictive or apoptotic routes, whereas in spermatogonia they do not interfere with their ability to proliferate. It is rather their depletion that leads to such cellular responses. A further issue, stemmed from the ability of ATM to regulate ARF turnover (Velimezi et al., 2013), is whether this functional link may operate in male germ cells. As demonstrated, in response to irradiation ARF protein in spermatogonia is markedly down-regulated (Velimezi et al., 2013). Evidence was also provided by Takubo et al. (2008) showing that in ATM null spermatogonia there is a higher activation of ARF, supporting the interconnection between ATM and ARF. Nevertheless, details on how this link endorses this developmental process require further clarifications.

ARF AS A REGULATOR OF THE VASCULAR NETWORK IN DEVELOPMENT AND TUMORIGENESIS

Apart from the male germ cell development in mice as presented above, ARF also plays a central role in the murine ocular development. In mice models it was shown that ARF is required for the maturation of the primary vitreous into the secondary vitreous; an avascular jelly like substance within the developing oculus. The expression of ARF in the vitreous is postnatally induced up to P5, in order to trigger the involution of HVS, a transient anatomical

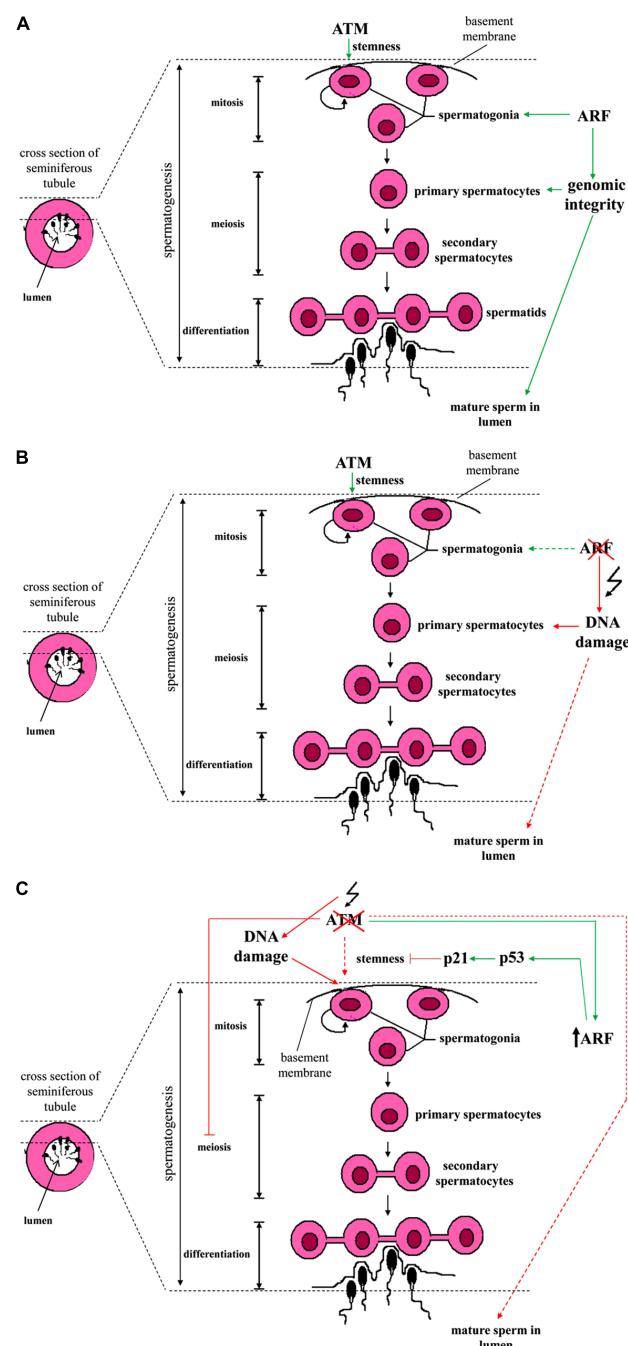


FIGURE 1 | Schematic presentation of the spermatogenesis stages across the wall of a seminiferous tubule and the interrelations with ATM and ARF. (A) Spermatogonia are found in close proximity to the basement membrane of a seminiferous tubule. A subpopulation of spermatogonia exhibits stem cell ability and self-renews via mitotic divisions. ATM kinase is essential for their stemness (symbolized by a semicircular shape). Some of the spermatogonia eventually differentiate into primary spermatocytes. The latter, undergo meiosis to give rise to secondary spermatocytes which in turn, form spermatids (connected via cytoplasmic bridges). Spermatids engage a series of cytodifferentiative programs and sperm is finally formed. ARF expression in spermatogonia is required in order to prevent the occurrence of DNA damage in meiotic primary spermatocytes (at the stage of pachytene) through participating in a feed-forward program. **(B)** In the absence of ARF, genomic integrity in

primary spermatocytes is threatened and the production of mature sperm released in the lumen is compromised. **(C)** Upon ATM deficiency, spermatogonia lose their genomic integrity and ARF undergoes upregulation. Consequently, an ARF-mediated p53/p21^{WAF1/Cip1} growth restrictive pathway counteracts spermatogonial stemness. Due to the fact that ATM deficiency hampers normal spermatocytic meiotic progression (at prophase I), sperm production is compromised (Barlow et al., 1996, 1998; Xu et al., 1996). The different subtypes of spermatogonial cells, Sertoli cells that support spermatogonia, stages and phases of meiosis, the different stages of spermatid differentiation as well as ploidy of cells are not shown here for reasons of simplicity (Colored lines depict ATM and/or ARF effects on spermatogenesis. Dashed colored lines denote weak effect or weak activation. Red lines represent adverse effect, while green ones correspond to physiologic functions).

entity in the developing oculus that has to regress during P6–P10 (McKeller et al., 2002). ARF promotes HVS involution through restricting the accumulation of mural cells that cover the vessels and contributes to the preservation of their stability in a PDGFR β -dependent/p53-independent manner (Silva et al., 2005; Gromley et al., 2009). Overall, two models have been proposed to explain the mechanistic basis by which ARF controls the vascular network dynamics. According to the first model, when ARF is induced by unknown yet upstream developmental signals it suppresses PDGFR β expression via uncharacterized mediators. In this way, ARF restricts mural cell proliferation. In the second scenario, ARF acts as a cell fate determinant during the maturation of mural cells to shut off PDGFR β expression and force them to differentiate into a type of perivascular cells that selectively support transient vessels (Thornton et al., 2005).

Notably, phenotypic characteristics exhibited by ARF deficient mice are also found in the developmental human ocular disease termed PHPV and include microphthalmia and degenerative alterations in lens (cataractogenesis; McKeller et al., 2002). Overall, these data pinpoint to the existence of an ARF-mediated tightly regulated spatio-temporal angiogenic developmental process. It appears that ARF's role in vascular evolution is not solely restricted to developmental processes. Rather, data corroborate the notion that ARF exhibits a wider activity in the control of vascular dynamics, which are functionally linked to tumor progression.

“Angiogenic switch” is a prominent feature of tumor progression (Bergers and Benjamin, 2003). In line with the notion that ARF plays a wide role in the modulation of pathways affecting vasculature, an inverse correlation among MVD and ARF in human clinical colon cancer samples has been reported (Kawagishi et al., 2010). Furthermore, ARF/p53 deficient MEFs challenged with oncogenic RAS^{V12} when injected as xenografts produce tumors that grow faster relative to similarly treated cells, but retrovirally infected with ARF (Kawagishi et al., 2010). Tumor sections showed in the latter case a lower immunostaining for CD31, a neovascular marker. Exploring mechanistically their findings Kawagishi et al. (2010) determined that ARF suppresses the expression of VEGFA in a p53-independent manner in mouse cell lines. This involves the inhibition of VEGFA translation via the IRES of VEGFA.

Based on our recent finding that ATM controls ARF turnover (Velimezi et al., 2013), we sought to expand these observations in human tumors with inactive p53 and explore for potential therapeutic utilization. Treating the NSCLC cell line H1299 and cervical carcinoma HeLa cells, which do not express functional p53, with the ATM kinase inhibitor Ku55933 in order to stabilize ARF (Velimezi et al., 2013) we observed an inverse relationship among the expression of ARF and VEGF protein (**Figure S1A**). VEGF plays a central role in tumor angiogenesis (Crinò and Metro, 2014), while ARF can signal in a p53-independent fashion and hence, serve as a “back-up” barrier to tumorigenesis in case that p53 is inactivated (Velimezi et al., 2013). Therefore, upregulating ARF via inhibition of ATM activity may be exploited in p53-deficient human tumors as a novel anti-angiogenic therapeutic approach. In a next step, we investigated whether ARF opposes tumor angiogenesis *in vivo*. To address this issue H1299

cells were xenografted in immunocompromised mice. Sections from the generated tumors were stained with an antibody specific for the endothelial marker CD31, to evaluate MVD. As shown in **Figure S1B**, MVD is markedly decreased (>twofold) in tumors injected with a lentiviral vector expressing shRNA targeting ATM (ctl-shRNA/Lenti-shATM); a manipulation which upregulates ARF (Velimezi et al., 2013). The observed reduction in MVD was actually an ARF-dependent phenomenon, because MVD in H1299-shARF xenografts upon knocking down of ATM (shARF/Lenti-shATM) was found to be comparable to that estimated in ARF-expressing ones without lentivirus-mediated silencing of ATM kinase (ctl-shRNA/Lenti-ctl). Therefore, stabilization of ARF in the absence of ATM activity exhibits anti-angiogenic effects *in vivo*, corroborating the claim that this p53-independent ATM/ARF axis could be therapeutically harnessed.

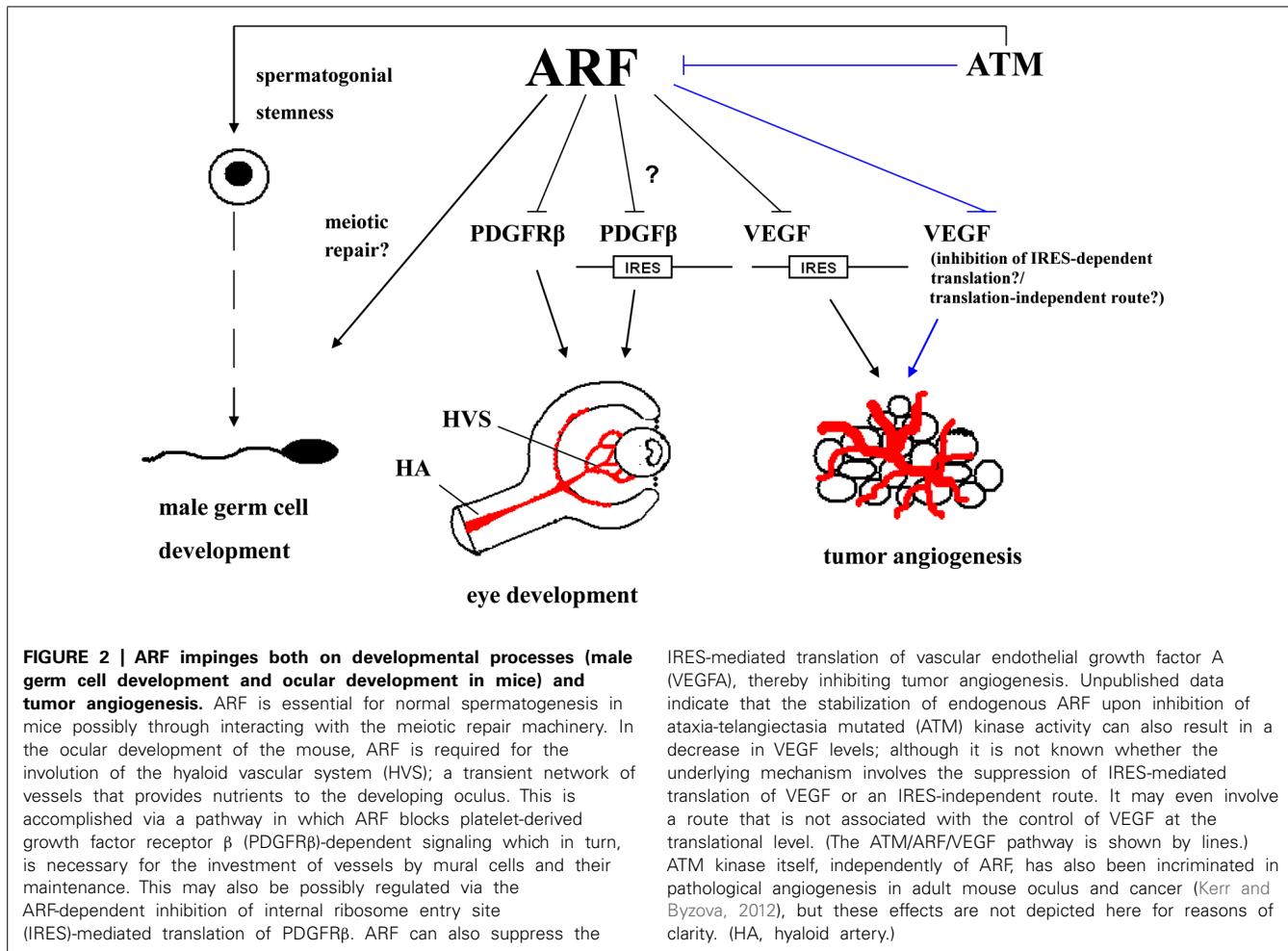
In our experiments we did not examine whether the observed ARF-mediated down-regulation of VEGF levels is associated with an inhibition of IRES-(in)dependent translation of VEGF transcript (Kawagishi et al., 2010), or if it is due to a translation-independent mechanism. For instance, HIF1 α is a well-known transcriptional activator of VEGF and human ARF has been demonstrated to mediate the nucleolar sequestration of HIF1 α , thereby hindering its ability to drive the expression of its target genes (Fatayol and Szalay, 2001). It would be a challenging future task to uncover the whole mechanistic spectrum that underlies the observed mutual exclusive expression among ARF and VEGF.

In a murine model of multi-stage pancreatic neuroendocrine tumorigenesis where the SV-40 T-antigen is expressed in β cells, ARF deficiency was found to significantly accelerate tumor progression through promoting the angiogenic switch (Ulanet and Hanahan, 2010). In the absence of ARF the tumor burden was increased fivefold along with a higher number of angiogenic lesions. From a mechanistic perspective, in this malignancy ARF seems to act via engaging mainly p53-independent routes, while VEGF was not involved (Ulanet and Hanahan, 2010). A more recent study showed that ARF blocks the development of angiosarcomas associated with the exposure to the carcinogen urothene, possibly in a p53-dependent fashion (Busch et al., 2012). Intriguingly, it was hypothesized that ARF affects the proliferation of endothelial cells in adult mice. Hence, the inhibition of tumor angiogenesis and vascular malignancy possibly represent two discrete aspects of ARF's tumor-suppressive activity.

CONCLUSIONS AND PROSPECTS

Collectively, we highlight underrated functions of ARF, positioned at the crossroads of tumor suppression (Weber et al., 2000; Tago et al., 2005; Kawagishi et al., 2010) and development (McKeller et al., 2002; Churchman et al., 2011; **Figure 2**), that could be exploited at the therapeutic level, especially in tumors with non-functional p53.

ARF as an onco-suppressor impedes carcinogenesis not only through interfering with cell proliferation and induction of apoptosis, but also via affecting other cancer-promoting processes such as angiogenesis (Kawagishi et al., 2010; Ulanet and Hanahan, 2010). In this context, in tumors with non-functional p53 the potential to upregulate ARF in an ATM manner



(Velimezi et al., 2013), could be utilized as an anti-angiogenic “tool” in cancer management (Figure S1). This prospective therapeutic modality may be enhanced if combined with anti-VEGF or other anti-angiogenic factors, like tyrosine kinase inhibitors, that are currently used (Ferrara et al., 2004; Randall and Monk, 2010; Eisen et al., 2012). On one hand, such a dual treatment might lead to a synergistic outcome, possibly lethality, and on the other hand, it may allow the reduction of the administration doses of such compounds to avoid side-effects (Randall and Monk, 2010; Eisen et al., 2012). It should be noted that although the ATM inhibitor Ku55933 is highly selective toward ATM, its bioavailability is low due to its pharmacokinetic properties (Golding et al., 2009). However, new ATM inhibitors, such as KU-60019, exhibiting a higher pharmacological profile have been released (Golding et al., 2009), rendering the proposed therapeutic approach feasible.

Another option in order to therapeutically exploit the aforementioned ARF/VEGF pathway would be the usage of synthetic ARF peptides comprising ARF’s amino-terminal residues 2–14 that mediate all the biological effects of ARF, including the anti-growth ones (Saporita et al., 2007). The therapeutic exploitation of the p53-independent ARF/VEGF axis is of major clinical importance since p53 is inactivated in \sim 50% of human cancers (Sherr, 2006).

A further aspect that needs to be addressed is the role of ARF in spermatogenesis. Deciphering the poorly defined ability of ARF to cross-talk with components of the HR mechanism during this process could provide new insights on other underrated functions of ARF. Specifically, in the case of male germ cells ARF contributes to their genomic integrity during their maturation, but the exact mechanism(s) is still lacking (Churchman et al., 2011). Even more, as ATM also participates in this process and since they are functionally linked (Velimezi et al., 2013), the exact way their function is coordinated throughout this process remains to be defined.

Finally, taking into consideration the role of ARF not only in the involution of HVS in the developing oculus (Thornton et al., 2005) but also in the involution of the mammary gland (Yi et al., 2004), it is plausible that ARF plays an even wider role than that of a tumor suppressor by acting as a potent “tissue remodeling factor” controlling transient histological structures. Studies toward this direction are essential and could open a new research field related with ARF.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://www.frontiersin.org/journal/10.3389/fgene.2014.00236/abstract>

Figure S1 | ARF demonstrates a p53-independent anti-angiogenic activity in a malignant environment. (A) Evidence for a p53-independent ATM/ARF/VEGF pathway. Human ARF protein (mouse monoclonal antibody, 1:100 dilution, DCS-240, #ab49166, Abcam) was up-regulated in the presence of the selective ATM inhibitor Ku55933 (Ku, #118500, Calbiochem, MERCK) and as previously shown biologically effective (Velimezi et al., 2013). This was associated with a decrease in VEGF protein (rabbit polyclonal antibody, 1:100 dilution, A-20, #sc-152, SantaCruz) levels as assessed by immunoblot analysis in human cancer cells H1299 and HeLa, both of which are devoid of functional p53. Actin (rabbit polyclonal antibody, 1:1000 dilution, Cell Signaling Technology Inc., #4967) served as a loading control. (ATMi: treatment with the Ku55933 chemical inhibition of ATM activity). **(B)** ARF-dependent decreased microvessel density in a malignant environment. The non-small cell lung cancer (NSCLC) cells H1299, which do not express a functional p53 were xenografted in immunocompromised mice and formed tumors (Velimezi et al., 2013). Immunohistochemical (IHC) analysis was carried out using an anti-CD31 antibody in order to evaluate mean microvessel density (MVD) in sections from tumor tissue. MVD is more than twofold decreased in tumors that were injected with a lentiviral vector expressing short-hairpin (sh) RNA targeting ATM (ctl-shRNA/Lenti-shATM) where ARF is known to be upregulated. On the contrary, the MVD in H1299-shARF xenografts, where ATM has been silenced (shARF/Lenti-shATM), is not decreased. Rather, it is comparable to those estimated in ARF-expressing xenografts without lentivirus-mediated silencing of ATM kinase (ctl-shRNA/Lenti-ctl).

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The ATM signaling network in development and disease

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The DNA damage response (DDR) rapidly recognizes DNA lesions and initiates the appropriate cellular programs to maintain genome integrity. This includes the coordination of cell cycle checkpoints, transcription, translation, DNA repair, metabolism, and cell fate decisions, such as apoptosis or senescence (Jackson and Bartek, 2009). DNA double-strand breaks (DSBs) represent one of the most cytotoxic DNA lesions and defects in their metabolism underlie many human hereditary diseases characterized by genomic instability (Stracker and Petrini, 2011; McKinnon, 2012). Patients with hereditary defects in the DDR display defects in development, particularly affecting the central nervous system, the immune system and the germline, as well as aberrant metabolic regulation and cancer predisposition. Central to the DDR to DSBs is the ataxia-telangiectasia mutated (ATM) kinase, a master controller of signal transduction. Understanding how ATM signaling regulates various aspects of the DDR and its roles *in vivo* is critical for our understanding of human disease, its diagnosis and its treatment. This review will describe the general roles of ATM signaling and highlight some recent advances that have shed light on the diverse roles of ATM and related proteins in human disease.

Keywords: ataxia-telangiectasia, Nijmegen breakage syndrome, AT like disease, ATM, Mre11 complex, apoptosis, senescence, DNA repair

THE DNA DAMAGE RESPONSE TO DOUBLE-STRAND BREAKS

In response to a diverse array of DNA lesions, cells mount a DNA damage response (DDR) to maintain genome integrity (Jackson and Bartek, 2009). Following the recognition of a DNA lesion by a sensor protein, the DDR sets in to motion a complex network of signal transduction. The DDR (**Figure 1**) controls cell cycle checkpoints, regulates transcription, recruits the appropriate DNA repair machinery to lesions, responds to metabolic requirements, and controls cell fate decisions, such as apoptosis and senescence. Ultimately, the DDR will prevent genomic instability from accumulating by preventing cells with damaged DNA from propagating or being passed on to progeny through the germline.

While cells must identify and respond to diverse lesions, the DNA double-strand break (DSB) represents a particularly important threat to genome integrity. DSBs can be generated by exposure to ionizing radiation (IR) or various chemical compounds, such as topoisomerase inhibitors, that interfere with DNA replication and cell division. More pertinent to the developmental pathologies of hereditary diseases arising from deficiencies in the DDR are endogenous sources of DSBs. The generation of the antibody repertoire as well as the maturation of germ cells both involve the programmed generation and repair of DSBs via cellular enzymes (Nussenzweig and Nussenzweig, 2010; Sasaki et al., 2010). DSBs can also arise during DNA replication due to exposure to metabolites, such as reactive oxygen species (ROS), the activity of enzymes, such as topoisomerases, which break and rejoin DNA strands, and limitations in raw material needed for replication, such as nucleotides, that can promote fragile site expression

and chromosomal breakage (Tsantoulis et al., 2008; Bester et al., 2011). Two major pathways of DSB are utilized in the cell, non-homologous end-joining (NHEJ), that is operative throughout the cell cycle, and homology directed repair (HDR) that is restricted to S/G2 when a sister chromatid is present as a template (for a detailed overview of these repair pathways and subpathways, we refer the reader to a recent review; Chapman et al., 2012). The killing of cancer cells via DSB generation is a major strategy in cancer treatment and the cellular responses and mechanisms of repair and acquired resistance to these agents is important to understand in order to improve the efficacy of current treatment regimens (Helleday et al., 2008).

DNA DOUBLE-STRAND BREAKS AND HUMAN DISEASE

The identification of numerous human genetic instability syndromes, as well as their modeling in different experimental systems, has been invaluable to our understanding of the DDR in human disease. Ataxia-telangiectasia (A-T, ATM mutation), the related A-T like disease (ATLD, MRE11 mutation), Nijmegen breakage syndrome (NBS, NBS1/NBN mutation) and the more recently identified NBS like disease (NBSLD, RAD50 mutation), all present with similar pathological outcomes in humans (Stracker and Petrini, 2011). Cells from these patients have increased levels of chromosomal instability, are highly sensitive to DSBs, and show defective signaling responses such as impaired checkpoint activation or variable defects in apoptosis. Patients are particularly affected in central nervous system (CNS) development, exhibiting either neurodegeneration or microcephaly, and display varying degrees of immunodeficiency (McKinnon, 2012). In

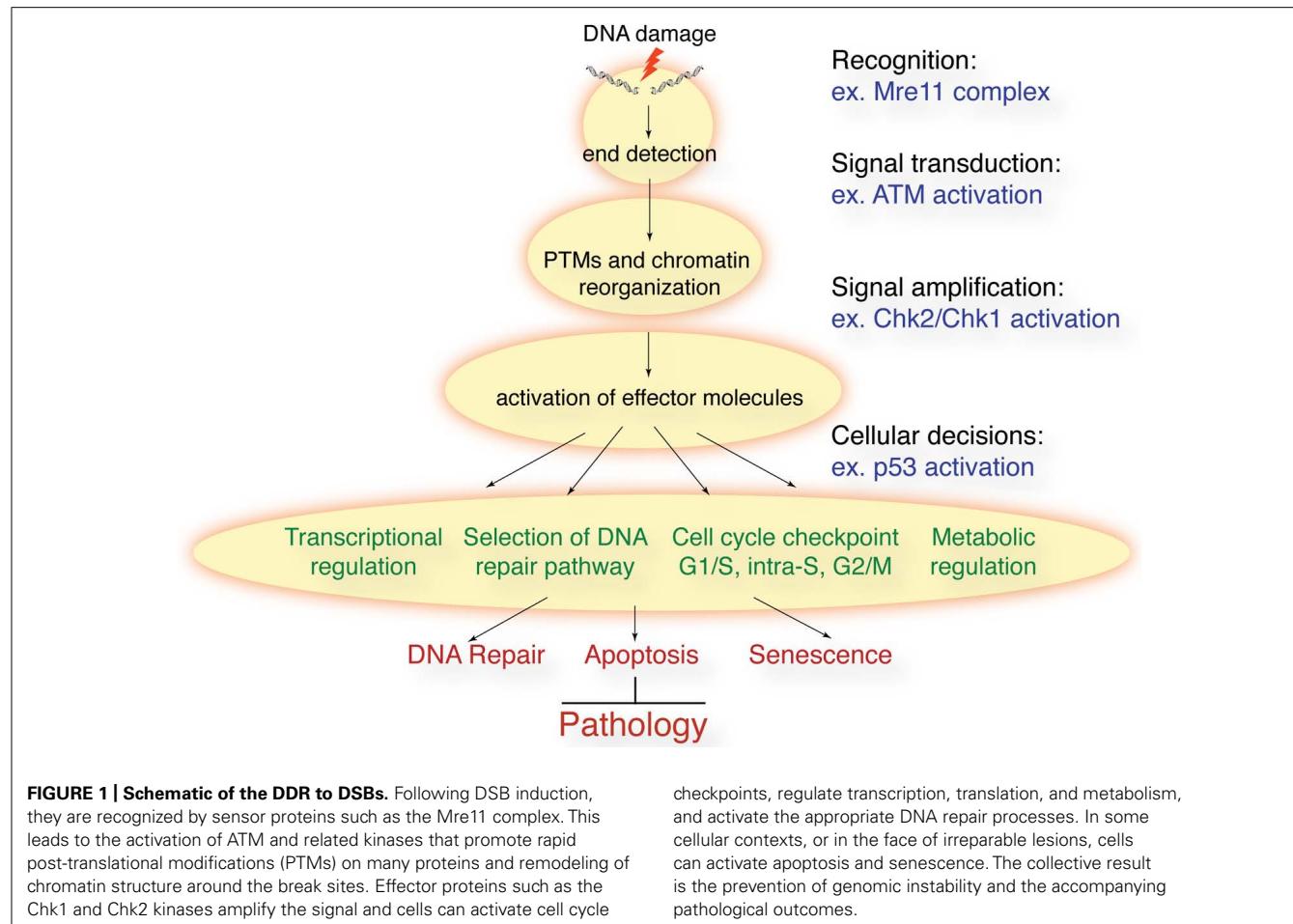


FIGURE 1 | Schematic of the DDR to DSBs. Following DSB induction, they are recognized by sensor proteins such as the Mre11 complex. This leads to the activation of ATM and related kinases that promote rapid post-translational modifications (PTMs) on many proteins and remodeling of chromatin structure around the break sites. Effector proteins such as the Chk1 and Chk2 kinases amplify the signal and cells can activate cell cycle

checkpoints, regulate transcription, translation, and metabolism, and activate the appropriate DNA repair processes. In some cellular contexts, or in the face of irreparable lesions, cells can activate apoptosis and senescence. The collective result is the prevention of genomic instability and the accompanying pathological outcomes.

addition, these disorders are often characterized by cancer predisposition and in some cases extensive problems related to fertility and metabolism. This review will focus on ATM kinase signaling and attempt to highlight recent work that has improved our understanding of its role in human disease through the regulation of DSB signaling and additional cellular functions that extend beyond the DDR.

ACTIVATION OF THE ATM KINASE: A CENTRAL TRANSDUCER OF DSB SIGNALING

ACTIVATION IN RESPONSE TO DNA DOUBLE-STRAND BREAKS

Double-strand breaks are recognized by the Mre11–Rad50–Nbs1 (MRN) or Mre11 complex, which is a sensor of DSBs. Capture of DNA ends by the Mre11 complex leads to the rapid activation of the ataxia-telangiectasia mutated (ATM) kinase (Stracker and Petrini, 2011). ATM is a member of the phosphatidylinositol 3-kinase-related kinase (PIKK) family and is the primary transducer of DSB-induced signaling (Lempainen and Halazonetis, 2009). The closely related disease pathology resulting from mutations in ATM, or any of the Mre11 complex genes, highlights their intimate relationship in DSB signaling. However, it is also worth noting that both ATM and the Mre11 complex have central functions independent from one another as ATM is synthetically lethal with many hypomorphic mutations in the Mre11 complex, some of which

do not impair ATM activation (Williams et al., 2002; Theunissen et al., 2003).

In undamaged cells, ATM exists in a dimeric or multimeric configuration (Bakkenist and Kastan, 2003). Following Mre11 complex sensing of DSBs, ATM undergoes autophosphorylation on at least four residues (S367, S1893, S1981, and S2996) that promote its monomerization and kinase activity (Figures 2A,B; Bakkenist and Kastan, 2003; Kozlov et al., 2006, 2011). Autophosphorylation is regulated through interactions with several phosphatases that exert opposing influences, including protein phosphatase 2A (PP2A), protein phosphatase 5 (PP5), and wild type p53-induced phosphatase 1 (WIP1; Ali et al., 2004; Goodarzi et al., 2004; Shreeram et al., 2006a). Human ATM-deficient cells complemented with S1981A, S367A, or S2996A mutants showed defective ATM-dependent responses to DNA damage (Kozlov et al., 2011). However, a murine allele with a triple mutation in the analogous sites to human S367, S1893, and S1981 complemented the defects of ATM deficiency *in vivo*, including checkpoint activity, germ cell development, lymphocyte development, and radiosensitivity (Daniel et al., 2008). Furthermore, autophosphorylation is not required for the activation of ATM in several *in vitro* settings (Lee and Paull, 2005; Dupre et al., 2006). The reasons for these discrepancies between complementation experiments with human and murine ATM remain unclear but may reflect species-specific

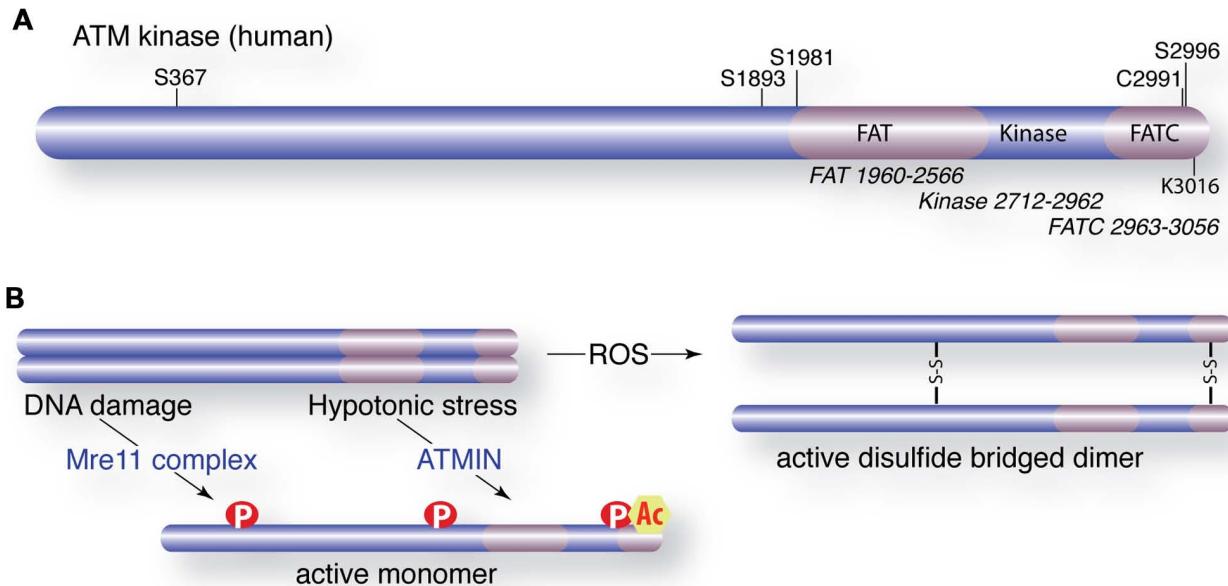


FIGURE 2 | Activation of ATM and post-translational modifications.

(A) Schematic of the ATM protein with domain organization (FAT = FRAP, ATM, and TRAP). Major autophosphorylation sites (S367, S1893, S1981, S2996), the TIP60 acetylation site (K3016) and a critical cysteine involved in ROS activation are shown. (B) Activation of ATM by DNA damage or

hypotonic stress requires the Mre11 complex (Mre11, Rad50, Nbs1) or ATMIN, respectively. Activated ATM is monomeric, phosphorylated and acetylated. Alternatively, ATM is activated directly by ROS that oxidizes cysteine residues to promote disulfide bridge-mediated dimerization.

differences or the experimental context (a recent review from the Khanna and Lavin groups included a detailed discussion of this issue; Bhatti et al., 2011).

The modulation of chromatin structure influences radiosensitivity and it has become clear that chromatin status plays a major role in ATM activation and the regulation of its activity at break sites (Bakkenist and Kastan, 2003; Murga et al., 2007). Acetylation of lysine 3016 by the TIP60 acetyltransferase is required for ATM activation in response to DSBs (Sun et al., 2005). TIP60 activity is dependent on activating transcription factor 2 (ATF2) and an interaction with histone H3 lysine 9 tri-methylation (H3K9me3) that is unmasked following the damage-induced removal of heterochromatin protein 1b (HP1b; Bhoumik et al., 2005; Sun et al., 2009). The Mre11 complex is crucial for the localization of TIP60 to H3K9me3, a well-established marker of heterochromatin that also occurs in other regions of the genome. ATF2 interacts with TIP60 and independently controls its levels and activity prior to damage. Dissociation of ATF2 following damage results in higher levels and activity of TIP60 to promote ATM activation (Bhoumik et al., 2008).

In addition to promoting ATM activation, the Mre11 complex, in conjunction with mediator of DNA damage checkpoint protein 1 (MDC1) mediator protein and the high mobility group protein HMGN1 (high mobility group nucleosome binding domain 1), facilitates the chromatin retention of ATM following DSB detection. The Mre11 complex promotes the rapid loading of active, phosphorylated ATM at breaks through interactions with the C-terminus of Nbs1 (Falck et al., 2005; You et al., 2005, 2007). ATM interactions with chromatin are enhanced in cells

treated with histone deacetylase inhibitors or in cells lacking HMGN1, arguing that chromatin topology plays a crucial role in mediating ATM–chromatin interactions, even in the absence of DSBs (Kim et al., 2009). Despite enhanced chromatin retention of ATM in cells lacking HMGN1, DSB-induced activation of ATM is impaired, consistent with the proposition that ATM interactions with chromatin prior to DNA damage govern its activation.

POST-TRANSLATIONAL MODIFICATIONS OF ATM

The mechanism by which post-translational modifications (PTMs) regulate ATM activation and activity remains largely unclear. It has been proposed that autophosphorylation sites may have cell cycle-specific roles or regulate other modifications that are required for ATM activation (Kozlov et al., 2011). The phosphorylation of ATM at S794 by cyclin-dependent kinase 5 (CDK5) in post-mitotic neurons has been reported to be required for subsequent autophosphorylation of S1981, providing some precedence for sequential regulation of ATM (Tian et al., 2009). The N-terminus of ATM has been identified as an interaction domain with Nbs1 and is required for chromatin retention (Falck et al., 2005; Young et al., 2005). Different patterns of phosphorylation in the N-terminus could modulate substrate interactions, chromatin binding, or subcellular localization. The damage-induced autophosphorylation of ATM on S1981 has been demonstrated to promote the retention of ATM to DSBs in a manner that is dependent on the Mre11 complex and the mediator protein, MDC1 (So et al., 2009). Collectively, existing data would support a model that auto and *trans*-phosphorylation could modulate

kinase activity, protein–protein interactions, substrate specificity, localization, and chromatin retention of ATM at DSB sites.

ATM ACTIVATION IN RESPONSE TO CELLULAR STRESS

In addition to DSBs, other types of cellular stress activate ATM in an Mre11 complex-independent manner (Bakkenist and Kastan, 2003; Guo et al., 2010). The ATM INteracting (ATMIN) protein (also known as ASCIZ or ZNF822) was identified as a mediator of ATM activation in response to hypotonic stress or chloroquine treatment (Figure 2B; Kanu and Behrens, 2007). ATMIN colocalizes to sites of DNA damage with phosphorylated ATM but deletion of ATMIN does not impair ATM activation or activity following IR treatment. Recent work from Behrens and colleagues has provided compelling evidence that ATMIN and Nbs1 compete for ATM binding as deletion of either protein enhances ATM signaling through the other (Zhang et al., 2012). Strikingly, they showed that the deletion of ATMIN rescued the proliferative defects and premature senescence of Nbs1-deficient cells, suggesting that the loss of Nbs1 resulted in ATMIN-ATM-mediated activation of p53 signaling. Murine cells expressing hypomorphic mutants of Nbs1 that lack the C-terminal ATM interaction domain of Nbs1 ($Nbs1^{\Delta C}$), a candidate domain for competition with ATMIN, showed normal ATM activation, in conflict with cell line complementation and biochemical data (Falck et al., 2005; You et al., 2005; Difilippantonio et al., 2007; Stracker and Petrini, 2011). One possibility is that ATMIN provides a redundant function in murine cells, where mutant Nbs1 is expressed at physiological levels, that is obscured by the overexpression of mutant forms of Nbs1 in the complementation of human cell lines. A more complete mechanistic understanding of how Nbs1 and ATMIN acquire the attention of ATM and affect its activity will no doubt provide important insights.

ATM ACTIVATION IN RESPONSE TO REACTIVE OXYGEN SPECIES

Increased ROS has been observed in ATM-deficient tissues and accumulating evidence suggests that this is highly relevant to A-T pathology. Recent work from the Paull lab has demonstrated direct activation of ATM by exposure to ROS *in vitro* (Guo et al., 2010). They proposed that ROS activates ATM by promoting the formation of disulfide bridges involving multiple cysteine residues, including conserved cysteine C2291 in the C-terminal FATC (FRAP, ATM, TRRAP C-terminal) domain (Figure 2B). The implications of this mode of activity are very exciting and raise the possibility that this active form of ATM may engage a different set of substrates. Again, how this is coordinated with Mre11 complex or ATMIN-dependent activation in the context of complex cellular stresses will be of great interest and have a potentially high impact on our understanding of ATM regulation and its role in human genetic instability disorders.

ATM SUBSTRATES AND CELLULAR FUNCTIONS

As ATM is a kinase, its primary role in the DDR is thought to be the phosphorylation of proteins that control signal transduction in response to cellular stresses, such as DSBs and ROS. To date, roughly 1000 proteins have been identified as potential ATM substrates using different approaches (Lavin and Kozlov, 2007; Matsuoka et al., 2007; Mu et al., 2007; Bensimon et al., 2010; Bhatti

et al., 2011; Choi et al., 2012). While many bona fide ATM targets have been identified, some of these can be modified by other PIKKs in response to different stress inputs or if ATM is absent. This overlap in substrate specificity has made connecting a particular PIKK to specific targets a formidable challenge in the field. Complicating this is the severe genetic interactions between ATM and DNA-dependent protein kinase catalytic subunit (DNA-PKcs), making the propagation of double mutant cells impossible, as well as the fact that ATR (ATM and Rad3-related) is an essential gene (Brown and Baltimore, 2000; Sekiguchi et al., 2001; Gladdy et al., 2006). Large-scale mass spectrometry in combination with kinase inhibitors has been employed with success and will no doubt be a useful, though imperfect, approach for future studies to identify both direct and indirect targets (Bensimon et al., 2010; Choi et al., 2012). While recent large-scale approaches have broadened the potential roles of ATM in the DDR, and many functions beyond it, its well characterized substrates reflect important regulatory roles in cell cycle progression, DNA repair and the control of cell fate, consistent with the cellular phenotypes of cells lacking ATM.

ATM AND CELL CYCLE CHECKPOINT REGULATION

A primary role of ATM in the DDR is the activation of cell cycle checkpoints throughout the cell cycle. Defective checkpoint activities were observed in cells from A-T patients over 20 years ago and were speculated to be a major contributor to radiosensitivity (Painter and Young, 1980). Since then, many ATM targets that are critical for checkpoint activation have been identified. These include the tumor suppressor p53, the cohesin subunit SMC1 (structural maintenance of chromosomes proteins), ATM, the Mre11 complex and additional enzymes involved in the activation of the related ATR kinase and checkpoint kinase 1 (Chk1) through the generation of single-stranded DNA (ssDNA) tails (Shiotani and Zou, 2009; Stracker and Petrini, 2011).

THE G1/S CHECKPOINT

Cells that experience DNA damage in G1 are prevented from entering S-phase by the G1/S checkpoint that is dependent on the activity of the p53 and has been clearly linked to tumor suppression (Massague, 2004). P53 is one of the first ATM targets to be identified and ATM-deficient tissues and cells show a strong defect in the stabilization of p53 following DNA damage (Siliciano et al., 1997; Banin et al., 1998; Canman et al., 1998). ATM phosphorylates p53 on S15 (S18 in mice) and in conjunction with additional modifications, contributes to p53 stability (Chao et al., 2006). The G1/S checkpoint is impaired in ATM-deficient cells although to a lesser extent than those lacking p53 where the defect is complete (Xu et al., 1998).

Both ATM- and p53-deficient mice are prone to lymphomas that are characterized by complex chromosomal rearrangements in lymphocytes (Zhu et al., 2002; Deriano et al., 2011). As cells undergoing programmed rearrangements during V(D)J [variable (V), diversity (D), and the joining (J)] recombination should not enter S-phase until repair takes place, it is likely that defects in this checkpoint play a major role in predisposition to lymphoma (Danska and Guidos, 1997). Consistent with this, mice expressing mutant alleles of p53 that are competent for initiating the G1/S checkpoint have a much longer tumor latency and develop

a broader spectrum of non-lymphoma type tumors than p53 alone (Liu et al., 2004; Barboza et al., 2006). While this interpretation is attractive based on known data, the generation of mice expressing a mutant, non-acetylatable form of p53 ($p53^{3KR}$) calls into question the relationship between DDR signaling mediated by ATM and p53 and tumorigenesis. Mice homozygous for the $p53^{3KR}$ allele showed defective G1/S checkpoint responses as well as impaired apoptosis and senescence in response to DNA damage, but were not prone to rapid tumorigenesis (Li et al., 2012b).

THE INTRA-S PHASE CHECKPOINT

Cells in S-phase exposed to DNA damage activate the intra-S phase checkpoint, leading to a transient reduction in DNA synthesis and suppression of origin firing. Defects in this checkpoint are characterized by radioresistant DNA synthesis (RDS) that was used as a diagnostic tool and to identify complementation groups in A-T prior to the cloning of the ATM gene (Painter, 1981). Later work showed that RDS was also a feature of cells from NBS and ATLD patients, implicating the Mre11 complex in this checkpoint response (Stracker and Petrini, 2011). The intra-S phase checkpoint is controlled by parallel pathways that are activated by the Mre11 complex and ATM and the checkpoint kinases Chk1 and Chk2 that phosphorylate the CDC25A phosphatase, leading to the inhibition of CDK2 activity and origin firing (Falck et al., 2002). Nbs1, the SMC1 component of cohesin and ATM2 have been identified as a critical ATM targets in the intra-S checkpoint response (Kitagawa et al., 2004; Bhoumik et al., 2005; Difilippantonio et al., 2007; Stracker and Petrini, 2011). However, this checkpoint remains poorly defined at the mechanistic level and the implications of its dysfunction to human health remain unclear, as intra-S defects do not correlate with any severe pathological outcomes in animal models (Kitagawa et al., 2004; Difilippantonio et al., 2007; Stracker and Petrini, 2011; Foster et al., 2012).

THE G2/M CHECKPOINT

ATM plays a critical role in the activation of the G2/M checkpoint that rapidly prevents G2 cells from entering mitosis after DNA damage. ATM also prevents cells damaged in other phases of the cell cycle from accumulating in G2 at later time points (this is also commonly referred to as the G2/M checkpoint in the literature, although it is mechanistically and genetically distinct; Xu et al., 2001, 2002). Hypomorphic mutations in Mre11 complex alleles, or its depletion by viral proteins, impair ATM activation and the G2/M checkpoint (Williams et al., 2002; Carson et al., 2003; Theunissen et al., 2003). This is also true, although to a lesser extent, in cells with Nbs1 mutations that do not have a pronounced effect on ATM activation (Williams et al., 2002; Uziel et al., 2003). In addition, some mutations in the tumor suppressor BRCA1, an ATM substrate that interacts with the Mre11 complex, impair G2/M checkpoint arrest (Xu et al., 2001, 2002). The deletion of the checkpoint kinase Chk2 in any murine backgrounds with defects in the G2/M checkpoint, including Chk1 heterozygotes, and particular Mre11 complex or BRCA1 alleles, results in tumor predisposition, indicating that the G2/M transition is important for tumor suppression (McPherson et al., 2004; Cao et al., 2006; Stracker et al., 2008; Niida et al., 2010). Understanding how ATM orchestrates the G2/M transition will be important for elucidating its role in disease

etiology and will potentially uncover additional candidate proteins involved in oncogenesis (we refer the reader to a recent review of the G2/M transition for more detailed information; Kousholt et al., 2012).

ATM SIGNALING IN MITOSIS

Available evidence suggests that there is not a general damage-induced checkpoint response in mitosis. However, a basal “priming” response that involves ATM and DNA-PKcs is critical for the normal tolerance of mitotic DNA damage (Giunta et al., 2010). A notable exception is in *Xenopus* where the Costanzo lab identified XCEP63 as a target of ATM/ATR following mitotic DNA damage (Smith et al., 2009). XCEP63 is phosphorylated in an ATM/ATR-dependent manner leading to its detachment from the centrosome and impairment of spindle assembly and mitotic progression. Recently, mutations in human CEP63 were found to underlie autosomal recessive primary microcephaly (Sir et al., 2011). CEP63 was demonstrated to play a key role in the recruitment of CEP152, another centrosomal protein implicated in microcephaly, to centrosomes and the artificial tethering of CEP152 to centrosomes rescued many of the cellular phenotypes of CEP63 mutant cells (Kalay et al., 2011; Sir et al., 2011). Whether the loss of a mitotic DDR, centrosome assembly, or additional functions of CEP63 underlie CNS pathology in humans remains to be determined. The relationship between CEP63 and ATM will require further exploration as the ATM/ATR target site in XCEP63 is not conserved in higher mammals, although additional ATM/ATR consensus SQ/TQ sites have been identified by mass spectrometry.

In addition to regulating the mitotic DDR, ATM has recently been implicated in the regulation of the spindle assembly checkpoint (SAC) through interactions with the Aurora B kinase (Yang et al., 2011). In the absence of DNA damage, ATM was activated in mitosis by Aurora B through the phosphorylation of serine 1403 on ATM. The complementation of ATM-deficient cell cultures with S1403A mutants failed to restore the SAC in contrast to S1981A or wild type ATM expression. While the range of substrates targeted by ATM in mitosis has not been elucidated, the phosphorylation of the Bub1 kinase, a critical regulator of the SAC, by ATM appears to play a major role in ATM-mediated SAC activation. How Aurora B phosphorylation of ATM can initiate its DNA damage-independent activation remains unclear but will be important to understand with regards to the basic mechanisms of ATM activation as well as the role of ATM in promoting chromosome stability.

REGULATION OF DNA REPAIR AND TRANSCRIPTION IN THE CONTEXT OF CHROMATIN

Although much conflicting data has been reported, recent work has clearly defined multiple roles for ATM activity in regulating both major pathways of DNA repair (NHEJ and HDR) as well as transcription in the vicinity of sites of DNA damage through its activity on numerous chromatin substrates.

DNA RESECTION COORDINATES THE CELL CYCLE CHECKPOINT AND DNA REPAIR

A key feature of the intra-S and G2 checkpoint responses is the ATM-dependent activation of the ATR and Chk1 kinases through

the generation of ssDNA by DNA resection (Shiotani and Zou, 2009). Resection is the conversion of a double-stranded DNA (dsDNA) end to a ssDNA overhang by the nucleolytic removal of one strand in the 5'-3' direction. The generation of these overhangs is critical for both checkpoint activation and subsequent HDR-mediated repair. Recent work has elucidated a two-step model for DNA-end resection following Mre11 complex end capture (Mimitou and Symington, 2008; Zhu et al., 2008). In the first step, the Mre11 complex and the associated CtIP protein (Sae2/Ctp1 in yeast) initiate limited 5'-3' resection of the break ends. The identity of the nuclease activity (or more likely, activities) that catalyze this initial step of resection remains an essential question. Recent work from the Neale laboratory provided evidence that in the context of ends with protein blocked termini, Mre11 uses its endonuclease activity to nick the DNA and this is followed by exonucleolytic resection toward the end in a 3'-5' direction, consistent with the polarity of Mre11 activity (Neale et al., 2005). In concert, exonuclease 1 (Exo1) could act on the same strand in the opposite direction consistent with its 5'-3' polarity. This elegant bidirectional resection model may be generally applicable to DSB resection, although it is likely that significant redundancy exists, as Mre11 nuclease mutants have mild defects in resection in yeast and a normal G2/M checkpoint in murine cells (Buis et al., 2008; Mimitou and Symington, 2009).

In a second step, additional nucleases, such as Exo1 or DNA2, carry out processive 5'-3' resection resulting in longer single-stranded tails. These ssDNA tails are bound by replication protein-A (RPA) and serve as a platform to recruit replication factor C (RFC)2–5, the 9–1–1 complex (Rad9, Hus1, Rad1), TOPBP1 and ATRIP that together facilitate the activation of the ATR and Chk1 kinases (Cimprich and Cortez, 2008). In addition to RPA, the single-stranded binding protein complexes SOSS1 and SOSS2 have been implicated in the recruitment of the Mre11 complex, ATM activation and resection via Exo1 (Richard et al., 2008, 2011a,b; Huang et al., 2009; Yang et al., 2012). However, the deletion or depletion of the crucial SOSS1 or SOSS2 components (Obfc2b/SSB1 or Obfc2a/SSB2) in mice does not recapitulate these phenotypes suggesting that they may be context or cell type specific (Feldhahn et al., 2012).

ATM GOVERNS DNA REPAIR IN HETEROCHROMATIN VIA KAP1

The modulation of chromatin structure is important for DNA repair processes, including resection, particularly in highly condensed regions such as heterochromatin. Recent genome wide studies have demonstrated that mutations in cancer cells occur more frequently in heterochromatin, suggesting that their repair poses a particular challenge to the cell that is relevant to tumorigenesis (Schuster-Bockler and Lehner, 2012). ATM facilitates the relaxation of heterochromatin through phosphorylation of the KAP1 (also known as TIFβ/FRIP1/TRIM28) protein (Ziv et al., 2006; Goodarzi et al., 2008). Depletion of KAP1 by short hairpin RNA (shRNA) rescued the radiosensitivity of lines treated with ATM inhibitor and the complementation of these lines by a non-phosphorylatable KAP1 mutant caused a repair defect, even when ATM was not inhibited. These and other results suggest that the relaxation of chromatin via Kap1 is a major pathway by which ATM regulates radiosensitivity and

repair in heterochromatin (the role of ATM and KAP1 in heterochromatin is discussed in detail in a recent review (Goodarzi and Jeggo, 2012)).

REGULATION OF DSB REPAIR THROUGH RNF20

ATM targets numerous ubiquitin ligases to coordinate transcription and repair following DNA damage. The Shiloh and Komatsu groups both identified the RNF20–RNF40 heterodimer, which normally monoubiquitylates H2B to promote transcription, as an ATM target required for DNA repair through both the HDR and NHEJ pathways (Moyal et al., 2011; Nakamura et al., 2011). The depletion of RNF20 resulted in radiosensitivity and aberrant localization of numerous DDR proteins, including XRCC4, Ku80, and Rad51, which could not be rescued by a non-ubiquitylatable H2B mutant. Monoubiquitylation of H2B is known to occur during transcription where it is followed by the methylation of histone H3K4 and H4K79, the former being required for the recruitment of the SNF2 (sucrose non-fermenting 2) chromatin remodeling protein. While the reports differ in their approaches and assessment of methylation following DNA damage, it was demonstrated using ChIP, under conditions that favored HDR, that H3K4 methylation and SNF2h (sucrose non-fermenting 2 homolog) recruitment occurred at break sites in an RNF20-dependent manner and that the small interfering RNA (siRNA) depletion of RNF20 and SNF2h was epistatic (Nakamura et al., 2011). Collectively, these works have led to the proposition that the ATM-dependent DDR reappropriates the cellular transcriptional apparatus to sites of DNA damage in order to promote chromatin remodeling and facilitate repair through multiple pathways (for a more elaborated perspective on this work we recommend to the reader a recent review from the Shiloh group; Shiloh et al., 2011).

ATM REGULATES RNAPI- AND RNAPII-DEPENDENT TRANSCRIPTION

Additional links between ATM, the ubiquitylation machinery and transcription was established by Greenberg and colleagues using a novel chromosomal reporter system. They showed that RNA polymerase II (RNAPII)-dependent transcription is silenced in kilobase regions surrounding DSBs (Shanbhag et al., 2010). Transcriptional silencing required ATM activity and was partially dependent upon the H2A ubiquitin ligases RNF8 and RNF168 that have been previously implicated in ATM-mediated chromatin relaxation (Ziv et al., 2006). While reminiscent of previous work that demonstrated that ATM inhibits RNA polymerase I (RNAPI) in a manner dependent upon the Mre11 complex and MDC1, the effects of ATM on RNAPII inhibition appear to be distinct (Kruhlak et al., 2007). The mechanistic details and additional ATM substrates that orchestrate this large-scale regulation of RNAPI and RNAPII, and the consequences of their dysfunction, will be important to determine. Notably, mutations in RNF168 underlie RIDDLE syndrome that has many overlapping pathological features with A-T (Stewart et al., 2009).

ATM AND THE REGULATION OF CELL FATE IN RESPONSE TO STRESS

Apoptosis, or programmed cell death, is essential for development, particularly in the immune system, and represents an important

mechanism for the clearance of cells with DNA damage. Apoptosis is triggered in response to a variety of DNA lesions, including DSBs, and defective apoptosis is considered a hallmark of cancer cells (Hanahan and Weinberg, 2011). Apoptosis is also relevant in the context of radio and chemotherapy used for cancer treatment. Cells in the gastrointestinal (GI) tract and the bone marrow undergo extensive cell death in response to DNA damage, potentially causing bone marrow failure and severe GI syndrome. Apoptosis as a response to DSBs is restricted to particular cell types and tissues, as most cell types in the adult do not undergo apoptosis. The exposure to genotoxic stress or the inability to repair persistent DNA damage can also lead to cell death through other mechanisms, such as mitotic catastrophe or necrosis, or the induction of cellular senescence. ATM plays key roles in regulating these cell fate decisions following genotoxic stress that can influence pathological outcomes.

ATM IN APOPTOSIS

Apoptosis in response to DSBs is regulated by p53 in many tissues, including lymphocytes. Stability of p53 is regulated through its phosphorylation by ATM and Chk2 as well as their regulation of ubiquitin ligases that control p53 levels, namely mouse double minute 2 (MDM2) and murine double minute X (MDMX) (Jenkins et al., 2012). The mutation of both ATM and Chk2 target residues in p53 ($p53^{S18/23}$ mice) to alanine results in apoptotic defects despite the fact that p53 is stabilized to normal levels (Chao et al., 2006). Apoptosis is impaired after DNA damage in both ATM- and Chk2-deficient cells and ATM–Chk2 double mutants have a stronger apoptotic defect, comparable to that of p53 null or $p53^{S18/23}$ animals (Figure 3; Stracker et al., 2007; Stracker and Petrini, 2008). Together, these and other data suggested that additional ATM/Chk2 targets besides p53 were required to regulate p53 stability and responses. Recent work from the Jones lab has demonstrated that the phosphorylation of MDM2 by ATM plays a central role in ATM-mediated p53 stabilization and its response to DNA damage (Gannon et al., 2012). Mice expressing a non-phosphorylatable mutant of MDM2 exhibited defects in apoptosis more severe than that seen in ATM deficiency and comparable to that of p53 null animals. This may suggest that this residue can be acted upon by other PIKKs in the absence of ATM. ATM-independent apoptosis in lymphocytes has been shown to require the DNA-PKcs protein using small molecule inhibitors or siRNA depletion (Callen et al., 2009). A possible scenario based on available data is that in the absence of ATM, DNA-PKcs can directly activate Chk2 and modify MDM2 to promote p53-dependent apoptosis (Figure 3).

The ability of ATM to activate apoptosis in thymocytes in response to radiation depends largely on the Mre11 complex. Mutations in Mre11 that impair ATM activation mimic the defective apoptosis phenotype of ATM-deficient cells (Stracker et al., 2008). Mutation of the C-terminal ATM interaction domain of Nbs1 also impairs ATM-dependent apoptosis without affecting ATM activation (Difilippantonio et al., 2007; Stracker et al., 2007). Cells lacking the Nbs1 C-terminus show deficient ATM phosphorylation of SMC1 and the proapoptotic BH3 interacting-domain (BID) protein, but normal p53 phosphorylation and stabilization, indicating that this domain controls a subset of ATM targets

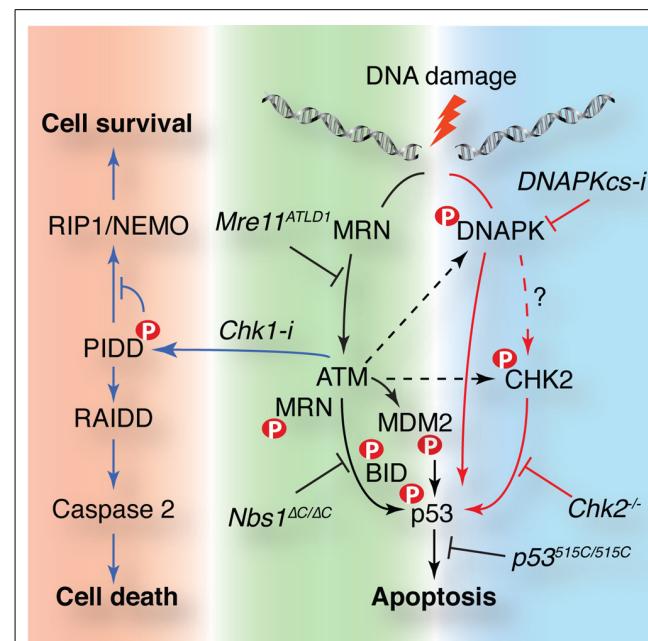


FIGURE 3 | ATM controls cellular survival in response to DNA damage through multiple pathways. DNA damage activates apoptosis in a p53-dependent manner in some cell types such as lymphocytes. p53 is activated by parallel pathways controlled by the Mre11 complex and ATM (black arrows) or DNA-PK and Chk2 (red arrows). The Mre11 complex is required for ATM activation and plays downstream roles mediating access to targets such as BID. Chk2 can act independently of ATM and may be a target of DNA-PK that is required for ATM-independent p53-dependent apoptosis. MDM2 phosphorylation by ATM is required for p53 stability and activity. ATM is also required for the Chk1 sensitized cell death pathway. ATM phosphorylates p53-induced protein with a death domain (PIDD) when Chk1 is inhibited favoring RIP associated Ich-1/CED homologous protein with death domain (RAIDD) binding that promotes caspase 2 activation and cell death. Murine alleles or small molecules (Chk1 or DNA-PKcs inhibitors) that affect these pathways are noted in italics. ATM targets are indicated with a red P.

(Kamer et al., 2005). How the C-terminus of Nbs1 influences ATM-dependent apoptosis remains unclear. As p53 is both phosphorylated (S18) and stabilized properly, it suggests that MDM2 is efficiently phosphorylated and that ATM can access p53 (Gannon et al., 2012). Nbs1 may be required for another critical modification of p53, such as acetylation, or the defect could be kinetic, caused by inefficient regulation of mitochondrial BID or another ATM substrate that is yet to be identified (Li et al., 2012b; Maryanovich et al., 2012).

Recent work has further elucidated the significance of ATM-mediated BID phosphorylation on cell fate and stem cell maintenance in the bone marrow. Previously, ATM has been implicated in regulating the self-renewal of hematopoietic stem cells (HSCs). ATM-deficient mice that escape lymphoma development exhibited progressive bone marrow failure that was rescued by treatment with antioxidants or the inhibition of p38 mitogen-activated protein kinase (p38MAPKs; Ito et al., 2004, 2006). Recent work from the Gross laboratory has identified BID as an ATM substrate and a regulator of HSC homeostasis (Kamer et al., 2005; Zinkel et al., 2005; Maryanovich et al., 2012). Mice expressing a mutant form of

BID (BID^{AA}) that is not modifiable by ATM were sensitive to radiation and showed impaired bone marrow regeneration. HSCs from these mice were less quiescent, exhibited higher levels of ROS and had increased levels of BID localized to the mitochondria. These results provide exciting links between the ability of ATM to regulate crosstalk between DNA damage, oxidative stress, and the HSC compartment (Maryanovich et al., 2012). It will also be interesting to determine if the Mre11 complex, that is required for efficient IR-induced BID phosphorylation, plays a role in BID-mediated HSC maintenance (Stracker et al., 2007).

As p53 is mutated in a high percentage of cancers, it is of therapeutic interest to identify p53-independent pathways of cell death that can be exploited to improve the efficacy of cancer treatments. Using zebrafish and mammalian cells, the “Chk1 suppressed” (CS) pathway of apoptosis was identified. Inhibition or depletion of Chk1 renders p53-deficient cells competent to induce cell death after DNA damage in an ATM/ATR and caspase 2 (CASP2)-dependent manner (Chen et al., 2009). Recent work from Sidi and colleagues has identified PIDD as an ATM target and critical component of this alternative cell death pathway (Ando et al., 2012). ATM modification of the PIDD death domain promotes its binding to RAIDD, rather than the prosurvival RIP1, to activate CASP2 and cell death (Figure 3). The physiological significance of this pathway, as well as the mechanism by which Chk1 prevents its activation, remain unclear but it will be of interest to further delineate these pathways as Chk1 inhibition in p53-deficient tumors is a promising strategy in clinical chemotherapy (Ma et al., 2012; Origanti et al., 2012).

ATM IN SENESCENCE

In addition to its role in cell death pathways, ATM has also been implicated in the regulation of senescence, although its role remains controversial. ATM-deficient fibroblasts in culture undergo senescence rapidly, likely due in part to the high levels of atmospheric oxygen used in standard culture conditions, as treatment with antioxidants or normoxic conditions prevent premature arrest (Parrinello et al., 2003; Ito et al., 2007; Woodbine et al., 2011). Thus, ATM inhibits senescence in response to oxidative stress. On the other hand, senescence induced by the overexpression of some oncogenes is dependent upon ATM (Bartkova et al., 2006; Mallette et al., 2007; Efeyan et al., 2009). As senescence has emerged as an important mechanism of tumor suppression and aging, it will be critical to clarify the importance of ATM in different contexts (Campisi, 2012). Recent work has elucidated a complex relationship between ATM signaling and the nuclear factor kappa B (NF- κ B) and p38MAPK pathways that can regulate cell survival, promote inflammation, and govern cellular senescence in response to DNA damage, ROS, and oncogene expression.

A consequence of senescence is the senescence-associated secretory phenotype (SASP) that can provoke defects in differentiation and promote tumor growth and invasion through the secretion of growth factors and inflammatory cytokines. DNA damage activates a SASP in primary fibroblasts that is dependent upon ATM, Nbs1, and Chk2 (Rodier et al., 2009). ATM regulates a subset of the SASP including IL-6 and IL-8. The Campisi group has demonstrated that the inhibition of the p38MAPK pathway blocks the

SASP following DNA damage independently of ATM and Nbs1 (Freund et al., 2011). Activity of p38MAPK activity is observed later than the initial DDR and is restrained by p53, suggesting that an attenuation of ATM and Chk2 pathways may be a prerequisite. Overexpression of a constitutively active p38MAPK activator MKK6 (MKK6EE) activated a SASP, demonstrating that high levels of active p38MAPK was sufficient. However, DNA damage induction was also required for optimal induction with several experimental systems that do not induce p38MAPK activation as strongly. The activation of the NF- κ B pathway was required for SASP induction downstream of p38MAPK and the authors have proposed that the DDR and p38MAPKs may co-regulate NF- κ B post-translationally to regulate the expression of its target genes and the SASP.

The NF- κ B pathway is activated in an ATM-dependent manner in response to some types of DNA damage (Wu et al., 2006). ATM phosphorylates the NF- κ B essential modulator (NEMO), leading to its ubiquitylation and the export of NEMO and ATM to the cytoplasm. NF- κ B signaling by ATM has been linked to immune system development, DNA repair, tumor progression, and defects in the nuclear lamina (Bredemeyer et al., 2008; Barascu et al., 2012; McCool and Miyamoto, 2012; Osorio et al., 2012). Work from the Lopez-Otin group recently described NF- κ B activation in an ATM- and NEMO-dependent manner in mice lacking the metalloproteinase Zmpste24 that processes prelamin A to lamin A (Osorio et al., 2012). This response underlies the progeroid features of mice lacking Zmpste24 that can be partially rescued by decreasing the gene dosage of lamin A.

Additional links between ATM and the nuclear lamina were uncovered by the Bertrand laboratory that found lamin B protein, but not mRNA, is overexpressed in ATM-deficient cells, leading to morphological defects (Barascu et al., 2012). Lamin B stabilization was dependent upon oxidative stress and a functional p38MAPK pathway. The mechanism by which ROS and p38MAPK promote lamin B stabilization remains unclear but impaired proteasome-mediated protein degradation caused by elevated expression of an ubiquitin-like protein ISG15 in the brain of A-T patients, as well as in ATM-deficient mice has been reported (Wood et al., 2011). ISG15 counteracts Ub-dependent proteasome degradation, and thus could provide a mechanistic explanation for the stabilization of proteins such as lamin B, as it targets many cytoskeletal proteins including lamin A (Zhao et al., 2005). How defects in the integrity of the nuclear lamina activate ATM remains unclear but as the lamin B receptor (LBR) has been linked to heterochromatin organization in the nucleus, and lamin B loss is a biomarker of senescence, understanding these connections will no doubt be informative (Goldberg et al., 1999; Freund et al., 2012).

ATM AND ONCOGENE-INDUCED STRESS

The overexpression of some oncogenes, such as c-Myc, can cause replicative stress, leading to an active DDR and the initiation of apoptosis or senescence depending on the cellular background. In the case of c-Myc, ATM and p53 have been identified as central mediators of c-Myc signaling (Campaner and Amati, 2012). Loss of ATM accelerates c-Myc-induced tumorigenesis in both an epithelial tumor (K5-myc) and lymphoma model (Eμ-myc) in

part by reducing apoptosis and augmenting proliferation (Pusapati et al., 2006; Maclean et al., 2007). Although it was not assayed as an endpoint in many previous studies, separation of function mutations in p53 implicate senescence as a major barrier to c-Myc-induced tumorigenesis in lymphocytes (Post et al., 2010). Consistent with this, deletion of the WIP1 phosphatase that restrains ATM and p53 activity, as well as both the NF- κ B and p38MAPK pathways, delays E μ -myc-induced tumorigenesis in a manner that requires both p53 and ATM (Bulavin et al., 2004; Shreeram et al., 2006a,b; Demidov et al., 2007; Chew et al., 2009). In contrast to the augmentation of c-Myc driven tumorigenesis in mice lacking ATM or p53, the mutation or inhibition of ATR leads to the converse outcome of impaired tumor development (Murga et al., 2011). These results highlight the inherent differences in the cellular roles of the ATM and ATR signaling pathways and have suggested that ATR pathway inhibition has potential as a chemotherapeutic strategy, particularly in oncogene addicted tumors (Toledo et al., 2011; Schoppy et al., 2012).

The influence of ATM deficiency on c-Myc-induced tumorigenesis is no doubt complex and involves multiple ATM targets. One target that may be of particular interest is the ubiquitin-specific protease 28 (USP28) that was identified as an ATM/ATR target through its interactions with 53BP1 and independently through a screen for genes required for Myc-induced transformation (Zhang et al., 2006; Popov et al., 2007b). USP28 has also been implicated in Chk2-dependent apoptosis as well as the maintenance of the ATM-dependent G2 checkpoint through the ability to stabilize the claspin protein (Bassermann et al., 2008). Based on these and other data, it has been proposed that DDR-induced phosphorylation of USP28 leads to its dissociation from the Fbw7 ubiquitin ligase complex, allowing unchecked Myc degradation, and its subsequent association with DDR components, such as 53BP1, claspin, Nbs1, and Chk2, to promote their stabilization (Popov et al., 2007a). This model predicts that mice or human patients lacking USP28 would fail to coordinate Myc levels with DNA damage signaling, potentially causing replication stress and enhanced genomic instability that could contribute to cancer (Shah et al., 2009).

DIVERSE ROLES OF ATM IN IMMUNITY

Ataxia-telangiectasia patients are more susceptible to infections and this has been attributed in part to varying degrees of immunodeficiency. Work in mice- and cell-based systems has identified a number of important roles for ATM in both adaptive and innate immunity, as well as inflammatory responses, that may underlie many aspects of pathology in A-T.

ADAPTIVE IMMUNITY

ATM plays an important role in the development of both T and B cells and A-T patients often exhibit abnormal T and B lymphocyte counts and deficient antibody responses. Lymphocytes are the main cellular component of the adaptive immune system that counteracts infections and cell abnormalities, including cancer. To react to a wide range of antigens, lymphocytes generate a diverse repertoire of antigen-specific receptors that depend on programmed chromosomal rearrangements that are initiated by enzymes that introduce DNA breaks. These processes, V(D)J

recombination (T and B lymphocytes) and class switch recombination (CSR, in B lymphocytes), are both dependent on intact ATM function. As A-T patients are prone to lymphomas, it is likely that the ability of ATM to monitor the development of lymphocytes through the regulation of both DNA repair and apoptosis plays a critical role in tumor suppression (Nussenzweig and Nussenzweig, 2010).

The lymphoid organs of mice lacking ATM are structurally intact but the absolute numbers of thymocytes are reduced due to developmental defects. The thymocyte population in ATM null mice is characterized by a reduction in mature single positive CD4 or CD8 T cells and an increase in immature double positive thymocytes (Barlow et al., 1996; Xu et al., 1996). Normal numbers of B cells are present in the spleen but a reduction in the number of B220 $^{+}$ IgM $^{-}$ Pre-B cells was observed in the bone marrow. Moreover, B cells are unable to respond properly to stimuli due to defects in B cell homeostasis and the regulation of programmed rearrangements during V(D)J and CSR. Recent work supporting this has demonstrated that ATM-deficient animals are able to stimulate T and B cell responses in response to chronic gammaherpesvirus infection, but that these responses are defective and unable to efficiently suppress viral replication (Kulinski et al., 2012).

V(D)J RECOMBINATION

During the development of T and B lymphocytes, V(D)J recombination is required for the assembly of antigen receptor genes. The recombination activating genes 1 and 2 (RAG1 and RAG2) constitute the RAG recombinase that generates DNA DSBs to catalyze recombination between the variable (V), diversity (D), and the joining (J) gene fragments in order to define the binding properties of the receptor (Boboila et al., 2012). NHEJ, one of the two major DSB repair pathways, is critical for the repair of RAG-induced breaks. The so-called “classical” or C-NHEJ pathway is defined as the DNA-PK holoenzyme (Ku70/80, DNA-PKcs), XRCC4, Lig4, Artemis, and XLF. The C-NHEJ pathway repairs the hairpin capped coding ends (CEs) and the blunt signal ends (SEs) generated by RAG activity. The kinase activity of DNA-PKcs stimulates the activity of the Artemis endonuclease that is required for the repair of CEs while the remaining C-NHEJ factors are critical for repair of both CE and SE ends. Blunt SE ends are repaired to a large extent in cells lacking DNA-PKcs or Artemis but not completely to normal levels (Boboila et al., 2012).

ATM colocalizes with RAG at endogenous recombination loci and RAG-induced DNA breaks persist in ATM-deficient cells due to incomplete defects in CE repair (Perkins et al., 2002; Callen et al., 2007; Nakamura et al., 2011). Persistent breaks in the immunoglobulin heavy chain (IgH) locus promote translocations, including those with Myc, that are known to promote lymphomagenesis and occur at higher rates in ATM-deficient cells (Ramiro et al., 2006). Recent work from the Alt lab has revealed an unexpected redundancy between ATM and other end-joining factors in the repair of both CE and SEs. Cells lacking both DNA-PKcs and ATM activity show a strong reduction in the level of SE repair (Zha et al., 2011b) and mice lacking both XLF and ATM exhibit reduced SE and CE joining (Zha et al., 2011a). Collectively, these and other studies suggest that ATM plays a role in monitoring

C-NHEJ-mediated repair during V(D)J recombination. This likely occurs through multiple activities that include the promotion of RAG complex stability, the activation of checkpoints to prevent the propagation of persistent breaks, the control of DNA end usage and the regulation of target proteins, such as H2AX and Artemis, that are shared with DNA-PKcs (Danska and Guidos, 1997; Bredemeyer et al., 2006; Callen et al., 2007; Bennardo and Stark, 2010; Deriano et al., 2011; Zha et al., 2011a).

CLASS SWITCH RECOMBINATION

In response to cytokine secretion or infections, B cells initiate antibody class switching (Boboila et al., 2012). This stimulation leads to rearrangements of the switch regions, thus altering the effector function of the antibody. Class switching is catalyzed by the activation-induced deaminase (AID) that promotes strand breakage. Human A-T patients frequently have impaired development of the immunoglobulin subtypes IgA, IgG2, IgG4, and IgE in the serum and ATM-deficient mice show strong defects in CSR (Lahdesmaki et al., 2000; Pan et al., 2002; Lumsden et al., 2004; Reina-San-Martin et al., 2004). In contrast, AID-dependent somatic hypermutation is not strongly affected by ATM deficiency (Pan-Hammarstrom et al., 2003; Lumsden et al., 2004).

Class switch recombination depends on the core C-NHEJ machinery but also has distinct requirements from V(D)J. The mechanism by which ATM regulates CSR is likely complex and represents a composite of the misregulation of many substrates. The ATM substrate 53BP1 is critical for both V(D)J and CSR where it has been proposed to play multiple roles including the synapsis of distal ends and the protection of free ends from DNA resection (Difilippantonio et al., 2008; Dimitrova et al., 2008; Bothmer et al., 2010; Bunting et al., 2010). The localization of 53BP1 to DNA breaks is dependent on H4K20 methylation and the PIKK-dependent phosphorylation of H2AX (Celeste et al., 2003; Bothmer et al., 2011). 53BP1 is phosphorylated on 28 consensus PIKK SQ/TQ target sites and the mutation of all of these residues to alanine impairs the ability of 53BP1 to support CSR and block resection, but does not prevent its localization to γH2AX containing breaks (Bothmer et al., 2011).

Recent work from the de Lange and Nussenzweig laboratories has shed light on the relevance of 53BP1 phosphorylation by ATM as they have demonstrated the PIKK consensus sites are required for its interactions with the RIF1 protein and the regulation of DNA resection (Jankovic et al., 2013; Zimmermann et al., 2013). In lymphocytes, the interaction between 53BP1 and RIF1 is ATM-dependent and the deletion of RIF1 leads to increased AID-dependent breaks in the IgH locus, an accumulation of cells in G2/M and extensive 5'-3' resection. The Casellas and Nussenzweig labs have linked the aberrant resection of AID-induced breaks in the absence of 53BP1 to lymphomagenesis using an innovative high-throughput sequencing approach that mapped AID-induced translocations as well as the asymmetric binding of the ssDNA binding protein RPA, allowing them to monitor resection (Jankovic et al., 2013). Further work will be required to understand precisely how ATM-deficient cells bypass the severe phenotypes of RIF1 and 53BP1 mutants in CSR, potentially through the redundant functions of other PIKK activities such as DNA-PKcs.

ATM IN FERTILITY

Due to the young age of A-T patients, fertility is often overlooked as a clinical issue. However, ATM plays critical roles in germline development as A-T patients present with gonadal dysgenesis and both male and female mice lacking ATM are infertile due to defects in meiotic progression (Barlow et al., 1996). Meiosis is the special cell division that ensures the formation of haploid cells, spermatozoa and ova, from diploid progenitor germ cells. Germ cells undergo two rounds of chromosome segregations after replicating their genomes only once. During the first meiotic division, DSBs are generated by the SPO11 protein and repaired by homologous recombination (HR; Keeney et al., 1997). HR leads to the synapsis of homologous chromosomes that are stabilized by the synaptonemal complex (SC), formed by the union of the two chromosomal core axes by a central element. Repair of DSBs in meiosis can lead to the formation of non-crossover (NCO, when a small region of the homologous chromosome is used as template to repair the damage) or crossovers (CO, when flanking regions around the DSBs are exchanged between the homologs). The formation of the right number of COs is critical to properly segregate the homologs in the first meiotic division and reduce the genome by half. In fact, inaccurate CO distribution in human oocytes is believed to be the major cause of human aneuploidy (Nagaoka et al., 2012).

ATM has a crucial role in the completion of mouse gametogenesis since both male and female ATM-deficient mice are sterile (Barlow et al., 1996). Testes and ovaries from ATM null animals display massive germ cell loss. While *ATM*^{-/-} spermatocytes arrest at the pachytene stage of meiotic prophase, *ATM*^{-/-} females lose all oocytes during the first days of life, before completing meiotic prophase (Barlow et al., 1998; Hamer et al., 2004; Di Giacomo et al., 2005). The cytological analysis of *ATM*^{-/-} spermatocytes revealed that ATM is required to complete homologous chromosome synapsis (Barlow et al., 1998; Pandita et al., 1999). SCs in ATM mutant spermatocytes are fragmented and this is dependent on DSB formation, as *Spo11*^{-/-} *ATM*^{-/-} spermatocytes do not show broken SCs (Bellani et al., 2005). Moreover, the ends of the fragmented axes from *ATM*^{-/-} spermatocytes contain recombination markers such as γH2AX, RPA, or RAD51 (Plug et al., 1997; Barchi et al., 2008).

As expected from the presence of these synaptic defects, recombination is compromised in *ATM*^{-/-} spermatocytes. H2AX phosphorylation, that arises as a response to programed DSB formation in early meiotic prophase, is delayed, implying that ATM is involved in the early steps of meiotic recombination (Fernandez-Capetillo et al., 2003; Barchi et al., 2005; Turner et al., 2005). Accordingly, the assembly of RAD51 foci is inefficient and mislocalized in *ATM*^{-/-} spermatocytes. While RAD51 foci colocalize with the SC in wild type cells, multiple foci also form in the chromatin of *ATM* mutant cells (Barlow et al., 1997, 1998).

The gonadal pathology of *ATM*^{-/-} mice is strongly influenced by the failure to complete meiotic recombination as it is partially rescued by the heterozygosity of *Spo11* that reduces DSB formation to 70–85% of wild type levels (Bellani et al., 2005; Barchi et al., 2008; Cole et al., 2012). In this background, *ATM*^{-/-} spermatocytes are able to complete meiotic prophase. Nevertheless, *Spo11*^{+/+} *ATM*^{-/-} spermatocytes are unable to complete meiotic recombination because markers of unrepaired breaks, like

phosphorylated H2AX, can be found even in cells that have completed meiotic prophase (Barchi et al., 2008). The persistence of DSBs or recombination intermediates normally provokes a checkpoint response that delays or stops meiotic progression, and can in some cases initiate programmed cell death (MacQueen and Hochwagen, 2011). In mice, DSB-dependent arrest in meiosis is believed to occur at the pachytene stage of meiotic prophase I (de Rooij and de Boer, 2003). However, despite exhibiting multiple unrepaired DSBs, *Spo11^{+/−} ATM^{−/−}* spermatocytes do not arrest at pachynema and progress to metaphase I (Bellani et al., 2005; Barchi et al., 2008). This uncoupling of DSB repair and meiotic prophase progression suggests that ATM could be required as part of a checkpoint mechanism that controls meiotic development in the mouse, a possibility that remains to be formally tested.

Heterozygosity of *Spo11* has also revealed that ATM is involved in CO control, as *Spo11^{+/−} ATM^{−/−}* cells have more COs than wild type cells (Barchi et al., 2008). Interestingly, *Spo11^{+/−} ATM^{−/−}* spermatocytes fail to form the obligate CO between the X and Y chromosomes, that contain a small region of homology that guarantees their proper segregation at the end of the first meiotic division. Furthermore, COs are more closely positioned in *Spo11^{+/−} ATM^{−/−}* mutants than in *Spo11^{+/−}* spermatocytes, suggesting that ATM not only is involved in controlling the number of COs formed, but also in their proper position and spacing, both crucial aspects for allowing proper homologous chromosome segregation. These studies reveal that ATM may have an important role in coordinating meiotic chromosome dynamics, including chromosome axis formation as well as recombination (Barchi et al., 2008). Thus, the tight relationship between SC defects and inefficient recombination in ATM-deficient cells suggests that ATM could coordinate these two mechanisms to ensure proper meiotic prophase progression in mouse spermatocytes.

Interestingly, mice with hypomorphic alleles of the *Mre11* or *Nbs1* genes exhibited meiotic defects similar to that of *Spo11^{+/−} ATM^{−/−}* mice. *Mre11^{ATLD1/ATLD1}* or *Nbs1^{ΔB/ΔB}* spermatocytes showed aberrant synapsis among homologous chromosomes, persistence of recombination markers until late meiotic prophase and altered number and location of COs (Cherry et al., 2007). These findings suggest that the Mre11 complex participates in meiotic recombination but more importantly, that most ATM functions during meiosis may be dependent on the Mre11 complex. Nevertheless, it is worth noting that, unlike *Spo11^{+/−} ATM^{−/−}* mice, both *Mre11^{ATLD1/ATLD1}* and *Nbs1^{ΔB/ΔB}* male mice are subfertile. This may imply that some of the meiotic activities of ATM are independent of the MRN complex function or that leaky ATM function in these Mre11 complex mutant backgrounds is sufficient for some degree of fertility (Theunissen et al., 2003).

Recently, the Jasin and Keeney labs reported that ATM is involved in controlling the number of meiotic DSBs created by Spo11 (Lange et al., 2011). Detecting the DNA oligonucleotides covalently linked to Spo11 as a readout of DSB formation, they showed that *ATM^{−/−}* mice have up to 10-fold more DSBs than wild type littermates (Neale et al., 2005; Lange et al., 2011). This was not due to differences in the cellularity of the mutant testis, as other mutants that arrest at the same stage as *ATM^{−/−}*, such as *Dmc1^{−/−}*, have approximately a 50% reduction in the amount of

Spo11-oligo complexes compared to wild type testis. They also showed that DSB formation was dependent on *Spo11* expression since *Spo11^{+/−} ATM^{−/−}* had fewer DSBs than *ATM^{−/−}* spermatocytes, but still significantly more than wild type cells. Moreover, cells expressing two extra copies of the *Spo11β* in an *ATM^{−/−}* background had even more DSBs than *ATM^{−/−}* spermatocytes, something that was not observed in an ATM proficient background. This work highlights the remarkable control of DSB formation in meiotic cells, revealing that it may be similarly deleterious for a meiocyte to form too few or too many DSBs. The authors propose that activation of ATM by DSB formation would create a local negative feedback loop that would inhibit *Spo11* activity in the vicinity of a DSB. This mechanism might be important to evenly space DSBs along the genome, in order to promote proper homologous synapsis, as well as minimizing the formation of DSBs in both sister chromatids in the same region of the genome, which could impair meiotic recombination.

ATM IN THE DEVELOPMENT OF THE CENTRAL NERVOUS SYSTEM

For reasons that remain largely unclear, brain development is highly susceptible to defects in the DDR. Unlike the immune system or germline, there are no known programs of DNA breakage and repair that would provide an obvious trigger for cell death. It has been speculated that mitochondrial defects, the accumulation of ROS, transposon mobilization, innate immune responses, the regulation of apoptosis or specific repair pathway defects may contribute to triggering neuronal cell death (Coufal et al., 2011; McKinnon, 2012; Petersen et al., 2012; Valentin-Vega and Kastan, 2012). One of the central pathologies associated with A-T is neurodegeneration, characterized by cerebellar atrophy and the loss of Purkinje and granule cells and subsequent ataxia. Notably, human diseases with the most similar neuropathology to A-T are caused by genes involved in the repair of diverse types DNA lesions (for more information on this topic, as well as the general neuropathology of A-T, we recommend to the reader this recent review; McKinnon, 2012). Unfortunately, neurodegeneration remains the most poorly understood aspect of A-T, as pronounced neurodegeneration and ataxia are not observed in mice lacking ATM. However, many insights have been gleaned from animal models and other systems that provide insight into the roles of ATM and the DDR in the CNS.

Central nervous system pathology is a feature common to many diseases caused by mutations in genes that encode members of the ATM-dependent DDR, including *MRE11* (ATLD), *NBS1/NBN* (NBS), *RAD50* (NBSLD), *RBBP8/CtIP* (Seckel), *ATR* (Seckel), *CEP63* (MCPH), *PNKP* (MCSZ), *TDPI* (SCAN1), and *ATM* (A-T; Das et al., 2009; Waltes et al., 2009; Segal-Raz et al., 2011; Sir et al., 2011; Stracker and Petrini, 2011; McKinnon, 2012; Poulton et al., 2012; Reynolds et al., 2012). However, depending on the particular mutation, patients, and in some cases, animal models, will develop either microcephaly (defined as a head circumference two-standard deviations smaller than the average) or neurodegeneration (defined as the progressive loss of neurons). It remains mechanistically unclear why mutations in *MRE11* result in neurodegeneration, similar to what is observed in A-T patients, while other mutations in *NBS1* or *RAD50* cause microcephaly.

Characterization of hypomorphic *Nbs1* and *Mre11* alleles in mice has revealed differential influences on the ATM-dependent DDR. The *Mre11^{ATLD1}* allele impairs ATM activation, thus reducing the total pool of active ATM and potentially affecting all substrates (Theunissen et al., 2003). In contrast, the *Nbs1^{ΔB}* allele does not impair ATM activation but reduces ATM activity on particular substrates (e.g., SMC1), while not affecting others (e.g., p53; Stracker et al., 2008). One clear phenotypic difference lies in the ability of these alleles to induce apoptosis following IR treatment (Stracker et al., 2007, 2008). Activation of p53-dependent apoptosis is impaired in the thymus and GI tract of *Mre11^{ATLD1}* animals but is indistinguishable from wild type in *Nbs1^{ΔB}*. Using both IR treatment and Lig4 deficiency to induce apoptosis in the developing brain, it has been demonstrated that this difference in apoptosis proficiency also applies to the CNS (Shull et al., 2009). An attractive possibility proposed from this work is that the status of apoptosis may dictate whether there is cellular attrition during development, leading to microcephaly, or whether cells experiencing genotoxic stress survive and are lost later due to other cell death pathways triggered by genomic instability, causing neurodegeneration. More recently, ATLD patients with microcephaly, rather than neurodegeneration, have been identified as well as NBSLD patients with microcephaly due to *RAD50* mutations (Waltes et al., 2009; Matsumoto et al., 2011). Determining if these *MRE11* or *RAD50* alleles impair apoptosis would provide a means for testing this proposition. The major question remains as to what triggers the DDR in the CNS and whether a few common or many diverse mechanisms are at play in the human diseases.

ATM deficiency is synthetically lethal with both the *Mre11^{ATLD1}* and *Nbs1^{ΔB}* alleles, hampering efforts to identify independent effects or potential redundancies in development (Williams et al., 2002; Theunissen et al., 2003). Deletion of any of the Mre11 complex members, including Nbs1, is embryonic lethal. Nevertheless, using conditional alleles, it has been demonstrated that CNS-specific loss of Nbs1 leads to microcephaly, defects in the development of the cerebellum and ataxia, in contrast to ATM-deficient mice or hypomorphic Nbs1 alleles that do not exhibit ataxia (Kang et al., 2002; Williams et al., 2002; Frappart et al., 2005; Stracker and Petrini, 2011). Many of these defects were rescued by loss of p53 and p53 activation was observed in CNS tissues where Nbs1 was deleted, suggesting that ATM activation may play a role in the pathological outcomes resulting from Nbs1 deficiency (Frappart et al., 2005). However, in contrast to p53 deficiency, loss of ATM exacerbated the microcephaly and ataxia phenotypes of CNS-specific Nbs1 deletion, again confirming that ATM and the MRN complex make independent contributions to CNS development (Dar et al., 2011). In addition, ATM-deficient mice with a deletion of Nbs1 in the CNS showed impaired growth and a markedly shortened life span. It would be interesting to compare the CNS of mice lacking both Nbs1 and ATM as ATM deletion elicits neurodegeneration and rescues other cellular phenotypes of Nbs1 null tissues (Kanu et al., 2010; Zhang et al., 2012). Whether ATM deletion would rescue CNS development by impairing ATM-mediated p53 activation or enhance the defects, as seen in mice lacking ATM, would be mechanistically informative.

One explanation invoked for the synthetic lethality of Mre11 complex mutations and ATM deficiency is the role of the Mre11

complex in activating ATR (Williams et al., 2002). Loss of ATR is embryonic lethal and hypomorphic mutations mimicking those found in human Seckel syndrome result in severe microcephaly in mice and are synthetically lethal with ATM deficiency (Brown and Baltimore, 2000; Murga et al., 2009; Ragland et al., 2009). The conditional deletion of ATR in the CNS also causes microcephaly and defective cerebellar development (Lee et al., 2012). However, in contrast to the deletion of Nbs1 in the CNS, the resulting pathology was not dependent on p53 (Murga et al., 2009). The balance of ATM and ATR signaling in the development of the CNS, and the organism as a whole, is clearly of great importance but remains poorly understood. Cleanly separating their functions and accounting for the potential redundancy of DNA-PKcs signaling remains an important challenge to overcome.

Recent work from the Herrup lab has linked ATM's role in CNS development to transcriptional regulation in neurons. They proposed that transcriptional defects caused by the aberrant nuclear localization of HDAC4 in ATM-deficient cells may contribute to neurodegeneration (Li et al., 2012a). Using a variety of approaches including ChIPseq, they showed that HDAC4 accumulated in the nucleus in ATM-deficient neurons and caused global defects in histone acetylation and neuronal gene expression. Of particular interest, nuclear HDAC4 suppressed the activity of myocyte enhancer factor 2A (MEF2A) and cAMP-responsive element binding protein (CREB) that control prosurvival programs. Treatment with histone deacetylase inhibitors reduced cell death and markers of cell cycle reentry in the ATM-deficient cerebellum. HDAC4 localization does not appear to be regulated directly by ATM-mediated phosphorylation but instead through ATM activity on the P65 subunit of PP2A, a known interactor of ATM (Goodarzi et al., 2004). ATM phosphorylation of PP2A promoted its cytoplasmic localization and prevented the dephosphorylation of HDAC4. The injection of mutant HDAC4 that localized only to the cytoplasm, coupled with shRNA downregulation of endogenous HDAC4, rescued some of the behavioral defects of ATM-deficient mice and reduced markers of cell death, providing a proof of principle for HDAC4 modulation in therapy. HDAC4 is expressed in many areas of the brain as well as other tissues, thus the pathological specificity of ATM loss on neurons remains unclear. It will be important to understand as well how HDAC4 is regulated in similar diseases resulting from Mre11 complex mutations to understand if this mechanism is particular to A-T or may have more widespread significance in genetic instability diseases.

THE ROLES OF ATM IN METABOLISM

Ataxia-telangiectasia patients exhibit several indices of metabolic disease including increased susceptibility to diabetes and impaired glucose metabolism. Some early indications for molecular roles of ATM in metabolism came from the identification of the translation regulator 4E-BP1 as an ATM target in response to insulin, the observation that ROS is increased in ATM-deficient animals and evidence for the peroxisomal localization of ATM (Barlow et al., 1999; Watters et al., 1999; Yang and Kastan, 2000; Kamsler et al., 2001). Subsequently it has been demonstrated that treatment with antioxidants could delay tumor formation in ATM null mice and rescue other aspects of development (Ito et al., 2004, 2007;

Schubert et al., 2004; Reliene and Schiestl, 2006, 2007; Reliene et al., 2008). The recent demonstration that ROS can directly activate ATM, that ATM can promote antioxidant responses through the stimulation of the pentose phosphate pathway (PPP) and evidence that ATM plays a role in monitoring mitochondrial quality control, has again pointed toward a central function of ATM in controlling cellular ROS metabolism (Guo et al., 2010; Cosentino et al., 2011; Valentin-Vega et al., 2012; these topics will be covered in brief here but we refer the readers to recent reviews/commentaries that cover some aspects in greater depth; Alexander et al., 2010b; Ditch and Paull, 2012; Valentin-Vega and Kastan, 2012).

Autophagy, the catabolism of dysfunctional or excess cellular components, is induced in response to diverse stresses including elevated ROS. In response to ROS, cytoplasmic ATM phosphorylated liver kinase B1 (LKB1) and activated AMP-activated protein kinase (AMPK; Alexander et al., 2010a). Together LKB1 and AMPK activated TSC2 that in turn repressed the mammalian target of rapamycin complex 1 (mTORC1) and induced autophagy. Recent work from the Kastan lab has shown that ATM-deficient thymocytes exhibited increased ROS and mitochondrial mass, as well as other markers of mitochondrial dysfunction (Valentin-Vega et al., 2012). Cells from ATM mutant animals have increased

basal autophagy but defects in mitophagy induced by mitochondrial membrane decoupling agents. A reduction in autophagy, by the deletion of one allele of the Beclin1 gene, reverted some of the mitochondrial phenotypes and significantly delayed tumor suppression without affecting the DDR, providing evidence that autophagy promotes tumorigenesis in the absence of ATM. In addition, ATM localized to mitochondrial fractions and was activated in response to mitochondrial membrane uncoupling. These and other data have led to the proposal that ATM may directly regulate mitochondrial homeostasis through responding to ROS or by regulating mitochondrial quality control genes, such as PINK1 or Parkin (Valentin-Vega and Kastan, 2012). As these proteins are mutated in Parkinson's disease, also characterized by CNS degeneration, understanding the regulation of mitochondrial integrity by ATM may shed light on the etiology of neurodegeneration in A-T.

Mitochondrial dysfunction can lead to excessive ROS generation that is a phenotype of ATM-deficient cells that has been described in many experimental settings, including those mentioned here in previous sections. Recent work from the Costanzo lab has defined a role for ATM in activating the PPP in response to DNA damage (Cosentino et al., 2011). ATM increased the activity

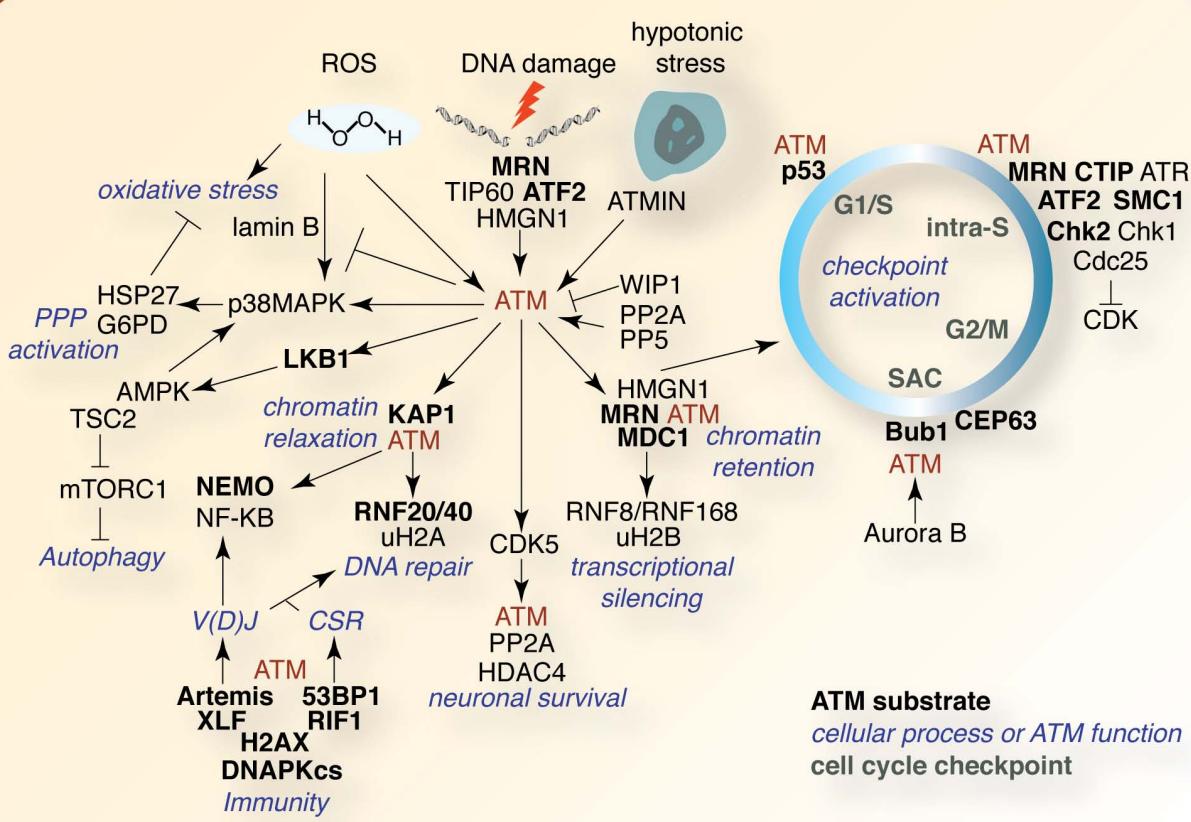


FIGURE 4 | Selected summary of the ATM signaling network. A non-comprehensive schematic of ATM signaling pathways described in the text. ATM substrates are indicated in bold black, other pathway effectors in black, affected cellular processes in blue, and cell cycle checkpoints in green. See text for details regarding the signaling pathways depicted.

of glucose-6-phosphate dehydrogenase (G6PD) by promoting its interaction with heat shock protein 27 (HSP27). This led to an increase in the production of nicotinamide adenine dinucleotide phosphate (NADPH), an essential co-factor for antioxidants and ribonucleotide reductase, as well as ribose-5-phosphate, that can be used for nucleotide biosynthesis. Interestingly, HSP27 is an important downstream target of the p38MAPK pathway and inhibition of p38 activity impaired the ATM-mediated stimulation of G6PD and phosphorylation of HSP27 on a target site regulated by p38MAPK (Cosentino et al., 2011). This indicated that p38MAPK activity was stimulated by ATM and required for ATM to promote an antioxidant defense through the PPP. In contrast, several reports have implicated p38MAPK activity in phenotypic outcomes resulting from increased ROS in the absence of ATM, including lamin B accumulation, impaired HSC homeostasis, impaired neural stem cell proliferation, cytokine secretion, and defective neoangiogenesis (Kim and Wong, 2009; Freund et al., 2011; Barascu et al., 2012; Maryanovich et al., 2012; Okuno et al., 2012). Understanding the complex relationship between ATM and p38MAPK signaling pathways will no doubt be of central importance in elucidating the mechanisms by which ATM modulates metabolism and suppresses a wide range of pathological outcomes.

Mice expressing hypomorphic Mre11 complex alleles recapitulate most DDR related aspects of ATM deficiency but are not tumor prone and do not exhibit the same oxygen-dependent phenotypes observed in the absence of ATM (Williams et al., 2002; Theunissen et al., 2003; Ito et al., 2004; Difilippantonio et al., 2005; Stracker et al., 2007; Valentin-Vega et al., 2012). It is therefore likely, at least in mice, that ROS signaling via ATM is largely intact and this could be sufficient for tumor suppression in Mre11 complex mutants. However, the clinical presentation in human patients of the same *Mre11^{ATLD1}* mutation modeled in mice is very similar to that of A-T, arguing against this possibility (Stewart et al., 1999; Theunissen et al., 2003). As so few ATLD patients with any one *MRE11* allele have been identified, it is difficult to definitively compare tumor predisposition, but as mitochondrial dysfunction and the response to ROS has been implicated as a major influence on the disease phenotypes, including neurodegeneration and tumorigenesis, it will be important to understand this aspect of ATM signaling and to what extent it is influenced or not

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CONCLUDING REMARKS

Our knowledge of ATM signaling in development and disease is ever expanding due to the creative work of dozens of labs using diverse experimental systems (Figure 4). The identification of specific ATM substrates and their functions will be instrumental in elucidating the mechanisms by which ATM can control so many critical cellular processes. This will require innovation at the level of sensitive high-throughput analysis of phosphorylation, and other PTMs, as well detailed single gene studies in available model systems. It is clear that the generation of animal models has been, and continues to be, invaluable to our understanding of ATM in tissue-specific processes such as apoptosis, CNS development, immunity, and angiogenesis, but they also do not faithfully recapitulate many crucial aspects of the human disease. Gene editing technologies, such as zinc-finger nucleases, should allow the manipulation of ATM and associated genes in higher organisms that may more faithfully recapitulate the human condition, particularly CNS pathology. Coupled with high-resolution sequencing and mass spectrometry-based technologies, including metabolic profiling, the pieces of the ATM puzzle will continue to fall into place and new strategies to exploit this knowledge can be used to benefit patients.

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Tumor protein 53-induced nuclear protein 1 enhances p53 function and represses tumorigenesis

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Tumor protein 53-induced nuclear protein 1 (TP53INP1) is a stress-induced p53-target gene whose expression is modulated by transcription factors such as p53, p73, and E2F1. TP53INP1 gene encodes two isoforms of TP53INP1 proteins, TP53INP1 α and TP53INP1 β , both of which appear to be key elements in p53 function. In association with homeodomain-interacting protein kinase-2 (HIPK2), TP53INP1 phosphorylates p53 protein at Serine-46. This enhances p53 protein stability and its transcriptional activity, leading to transcriptional activation of p53-target genes such as p21 and PIG3, cell growth arrest and apoptosis upon DNA damage stress. The anti-proliferative and pro-apoptotic activities of TP53INP1 indicate that TP53INP1 has an important role in cellular homeostasis and DNA damage response. Deficiency in TP53INP1 expression results in increased tumorigenesis, whereas TP53INP1 expression is repressed during early stages of cancer by factors such as miR-155. This review aims to summarize the roles of TP53INP1 in blocking tumor progression through p53-dependant and p53-independent pathways, as well as the elements which repress TP53INP1 expression, hence highlighting its potential as a therapeutic target in cancer treatment.

Keywords: TP53INP1, p53, protein phosphorylation, apoptosis, autophagy

INTRODUCTION

The TP53 gene encodes for the p53 protein which modulates target gene expression, regulates cell cycle progression and apoptosis, and functions as a tumor suppressor. p53 has been described as “the guardian of the genome,” due to its role in conserving stability by preventing the occurrence of mutation in the genome (Strachan and Read, 1999).

One of the key target genes of p53 is tumor protein 53-induced nuclear protein 1 (TP53INP1). TP53INP1 is expressed in many tissues upon exposure to various stress agents, and encodes two nuclear isoforms, TP53INP1 α and TP53INP1 β , both of which appear to be key elements in p53-mediated cell cycle arrest and apoptosis in different cell types (Tomasini, 2003). As a tumor suppressor, TP53INP1 has been reported to be down-regulated in cancers from different organs (Jiang et al., 2006; Gironella et al., 2007; Shibuya et al., 2010).

Tumor protein 53-induced nuclear protein 1 gene localizes to human chromosome 8q22 (Nowak et al., 2005), which shows sequence conserved with the A1–A2 of the murine chromosome 4 where the mouse TP53INP1 has been mapped (Carrier et al., 2000). Sequence analysis by the HUGO Gene Nomenclature Committee has revealed that stress-induced protein (SIP), p53-dependent damage-inducible nuclear protein 1 (p53DINP1), and thymus-expressed acidic protein (TEAP) are all in fact TP53INP1.

Tumor protein 53-induced nuclear protein 1 was first cloned and characterized in an attempt to identify pancreatic genes induced by the cellular stress acute pancreatitis in mouse, using a quantitative fluorescent cDNA microarray hybridization approach

(Tomasini, 2001). The mouse TP53INP1 gene is almost 20 kb pairs in length with five exons. The exon 4 of 28 base pairs is alternatively spliced to generate two transcripts which translates into two nuclear proteins of 18 and 27 kDa, SIP¹⁸ and SIP²⁷ (Tomasini, 2001) corresponding to TP53INP1 α and TP53INP1 β respectively (Tomasini, 2003). TP53INP1 α and TP53INP1 β proteins differ in their C-terminal region and can promote apoptotic cell death when overexpressed (Tomasini, 2001). Both TP53INP1 α and TP53INP1 β are rapidly and strongly induced in pancreatic acinar cells during the acute phase of pancreatitis and the exposure to various stress agents such as UV, DNA base damaging, ethanol, heat shock, and oxidative stress.

In this review, we aim to summarize the mechanisms through which TP53INP1 blocks tumor progression via p53-dependant and -independent pathways, and the mechanisms through which TP53INP1 gene expression is suppressed in cancer. Additionally, we will discuss the diverse functions of TP53INP1 in cancer such as induction of autophagy and repression of tumor cell migration, and highlight its potential as a therapeutic target in cancer treatment.

TP53INP1 FUNCTIONS AS A TUMOR SUPPRESSOR AND INDUCES APOPTOSIS THROUGH PHOSPHORYLATING p53 AT SERINE-46

Multiple lines of evidence suggest that TP53INP1 gene expression is modulated by p53. Firstly it has been shown that cells with deleted, mutated, or inactive p53 are unable to activate TP53INP1 gene expression in response to stress agents (Tomasini et al., 2002).

Secondly mouse embryo fibroblasts transformed with rasV12/E1A has shown stronger induction of TP53INP1 mRNA expression by activating p53-dependent pathway, compared to fibroblasts without p53 activity (Tomasini et al., 2002). Both observations suggest that TP53INP1 gene expression is activated by p53 in response to stress or transformation in cells expressing wild-type p53.

Tumor protein 53-induced nuclear protein 1 phosphorylates p53 at Serine (Ser)-46 by forming protein complexes with the protein kinase homeodomain-interacting protein kinase-2 (HIPK2) or protein kinase C δ (PKC δ) (Figure 1). HIPK2, a member of a novel family of nuclear serine/threonine kinases, co-localizes with p53 and PML-3 in the nuclear bodies and is activated after irradiation with ultraviolet. HIPK2 directly interacts with p53 and phosphorylates p53 at Ser-46, leading to p53-target gene transcription and the activation of p53-dependent apoptosis pathway (D'Orazi et al., 2001; Hofmann et al., 2002). Further analysis of subcellular distributions showed that p53, HIPK2, TP53INP1 α , and TP53INP1 β all localize into the pro-myelocytic leukemia nuclear bodies, PML-NB, which are cell cycle-regulated nuclear structures appearing as punctate foci in interphase nuclei (Tomasini, 2003). Such co-localization facilitates the protein interactions by positioning the resulting complex near its site of action (Tomasini, 2003). Importantly, both TP53INP1 α and TP53INP1 β in association with HIPK2 regulate p53 transcriptional activity on p21, PIG3, and BAX promoters, induce G1 cell cycle arrest and increase p53-mediated apoptosis (Tomasini, 2003).

In another study, Yoshida et al. (2006) have demonstrated that PKC δ , another kinase from the family of nuclear serine/threonine kinases, also associates with p53 and mediates its phosphorylation at Ser-46 upon exposure to genotoxic agents, hence promoting p53-mediated apoptosis in cellular response to DNA damage (Yoshida et al., 2006). Moreover, PKC δ functions as a protein kinase

and physically binds to TP53INP1 upon genotoxic stresses, leading to the formation of the PKC δ -TP53INP1 protein complex to regulate p53 protein phosphorylation at Ser-46 as well as p53-induced apoptosis (Yoshida et al., 2006).

The induction of growth inhibition and apoptosis is one of the most important tumor suppressive functions of p53. The challenge to find the exact mechanisms of p53-dependent apoptosis remains ongoing. In 2000, it was shown by Oda et al. (2000) that phosphorylation of p53 at Ser-46 by the p53-target gene; p53 regulated apoptosis inducing protein 1 (p53AIP1), could specifically regulate the induction of apoptosis. p53AIP1 was originally isolated as a p53-target gene using yeast enhancer trap system that allowed direct cloning of p53-binding sequence from human genomic DNA in order to isolate p53-target genes. p53AIP1 gene expression is strongly inducible by DNA damage in a p53-dependent manner, and is specifically induced after the phosphorylation of p53 protein at Ser-46, leading to apoptosis (Oda et al., 2000). Importantly, TP53INP1 induces p53 phosphorylation at Ser-46 and p53AIP1 expression, whereas the inhibition of TP53INP1 expression clearly impairs p53 phosphorylation at Ser-46 and p53AIP1 expression. Therefore, TP53INP1 is required for p53 phosphorylation at Ser-46, the induction of p53AIP1 and apoptosis (Okamura et al., 2001) (Figure 1).

As p53 is the most important tumor suppressor and p53 mediates cellular stress responses which are disrupted during tumorigenesis, it is important to further understand the mechanisms through which TP53INP1 interacts with HIPK2 and PKC δ kinases and phosphorylates p53 protein at Ser-46. As there are only handful studies demonstrating that TP53INP1 forms a complex with these two kinases to this date, it is important to understand what other co-factors are involved in the protein complexes and how these co-factors promote the formation of the

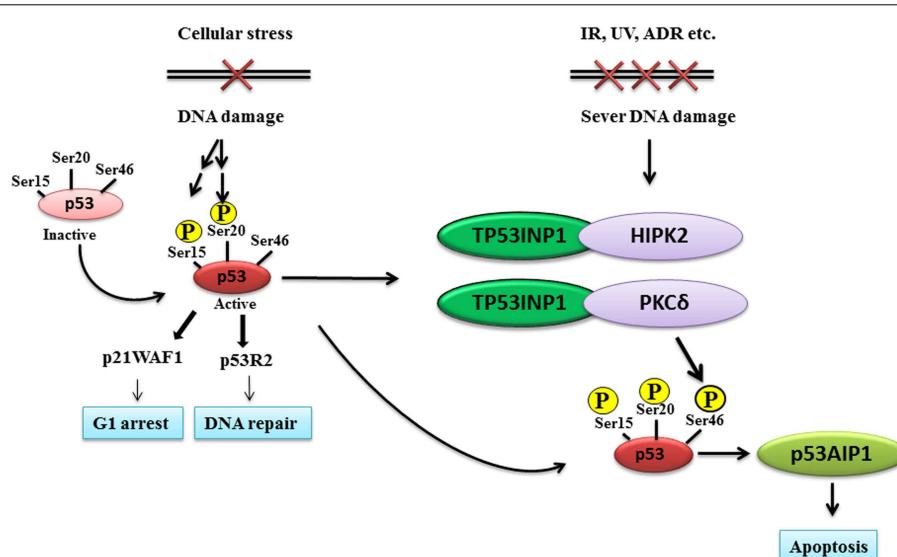


FIGURE 1 | TP53INP1 induces p53 protein phosphorylation at Ser-46 and apoptosis after DNA damage. Upon initial DNA damage, p53 is phosphorylated at Ser-15 and Ser-20, stimulating the binding of p53 to the promoter regions of a subset of genes such as the G1 arrest gene p21, the

DNA repair gene p53R2 and p53 negative regulators. If DNA damage is severe, TP53INP1 forms protein complexes with the Ser-46 kinase HIPK2 and PKC δ , leading to p53 protein phosphorylation at Ser-46, induction of p53AIP1 gene transcription and apoptosis.

protein complexes and the phosphorylation of p53 protein at Ser-46.

TP53INP1 INDUCES GROWTH INHIBITION AND APOPTOSIS IN A p53-INDEPENDENT MANNER THROUGH p73

It has been shown that in the absence of p53, TP53INP1 gene transcription can be strongly induced by p73, a p53 homolog. p73 is up-regulated in response to cisplatin, gamma-irradiation, and the oncogene E1A (Das et al., 2003; Hershko et al., 2005), activates p53-target gene transcription by binding to p53-responsive elements at p53-target gene promoters (Obad et al., 2004), and consequently induces cell cycle arrest and/or apoptosis (Jost et al., 1997; Kaghad et al., 1997). When p53-deficient mice are induced to suffer from acute pancreatitis or p53-deficient embryonic fibroblasts are treated with cisplatin, a strong DNA damaging agent, p73 activates TP53INP1 gene transcription, causes cell cycle arrest and apoptosis in a p53-independent manner (Tomasini et al., 2005). Using cells from p53-deficient mice, Tomasini et al. (2005) have demonstrated that the activation of the TP53INP1 gene promoter by p73 requires the presence of the p53-responsive element which is located between 1364 and 1239 base pairs upstream of TP53INP1 transcription start site. This suggests that p73 overexpression can directly activate TP53INP1 gene transcription through direct binding to the promoter region of TP53INP1.

Conversely, TP53INP1 alters the transactivation capacity of p73 on several p53-target genes, including TP53INP1 itself, demonstrating a functional association between p73 and TP53INP1 (Tomasini et al., 2005). Importantly, when overexpressed in p53-deficient cells, TP53INP1 activates p73, inhibits cell growth, and promotes cell death as assessed by cell cycle analysis and colony formation assays, hence the activation of TP53INP1 could potentially prevent tumor development (Tomasini et al., 2005). It is worth mentioning that TP53INP1 is able to stimulate p53 activity at much higher level compared to p73 activity.

INDUCTION OF TP53INP1 GENE EXPRESSION BY E2F1 CAN BE EITHER p53 DEPENDENT OR INDEPENDENT

E2F1 is a transcription factor, which induces apoptosis via both p53 dependent and independent mechanisms. E2F1 controls the expression of a vast number of genes that are essential for progression from G1 to S phase (Hanahan and Weinberg, 2011). A study by Hershko et al. (2005) have shown that excessive activity of E2F1 results in increased expression of TP53INP1 as well as several other co-factors such as the apoptosis stimulating proteins of p53 (ASPP) family member ASPP1 and ASPP2, and the pro-apoptotic JMY. Although it is well documented that E2F1 can up-regulate p53 expression (Hershko et al., 2005; Polager and Ginsberg, 2009), ectopic expression of E2F1 in p53-null human H1299 lung adenocarcinoma cells results in an increase in TP53INP1 mRNA (Zemskova et al., 2010). This indicates that E2F1-mediated up-regulation of TP53INP1 can occur in a p53-independent manner. Moreover, chromatin immunoprecipitation assays with primers targeting -415 bp to -121 bp region of the TP53INP1 gene promoter, confirms that E2F1 directly binds to TP53INP1 gene core promoter and activates TP53INP1 gene

transcription (Hershko et al., 2005). Given that E2F1 can play a major role in cell cycle progression and apoptosis, it could provide very important information to further investigate the underlying mechanisms responsible for E2F1-induced TP53INP1 expression.

MODULATION OF TP53INP1 EXPRESSION BY INFLAMMATORY MEDIATORS

Inflammation contributes to the tumor microenvironment by providing the tumor with essential factors for proliferation, survival, tumor cell migration, and invasion. Inflammatory mediators play important roles in tumor initiation and development in certain cancer types, such as prostate cancer (De Marzo et al., 2007). TP53INP1 expression has recently been found to be enhanced in prostate cancer cells after treatment with the pro-inflammatory mediators tumor necrosis factor α and interleukin 6, indicating that TP53INP1 overexpression could be involved in inflammation-mediated prostatic carcinogenesis (Giusiano et al., 2012). In this scenario, overexpression of TP53INP1 actually results in increased tumorigenesis, contradicting with the tumor suppressor characteristic of TP53INP1 as described in various other cancers, suggesting a tissue specific function. Perhaps, TP53INP1 can act either as a tumor suppressor gene or an oncogene depending on the tissue type or the tumor microenvironment.

Micro RNAs REDUCE TP53INP1 mRNA EXPRESSION

MicroRNAs (miRNAs) are a new class of small (21–23 nucleotides) non-coding RNAs. They function as post-transcriptional regulators of gene expression through base-pairing to complementary sites on their target mRNAs and are involved in carcinogenesis. Multiple lines evidences demonstrate that TP53INP1 expression can be regulated by miRNAs at the post-transcriptional level. For example, Yeung et al. (2008) have shown that the miRNAs miR-93 and miR-130b, which are up-regulated in HTLV-1-transformed human T-cell lines, target the 3' un-translated region (3'UTR) of TP53INP1 mRNA, and that knocking-down the miRNAs significantly increases TP53INP1 mRNA expression and reduces proliferation and survival of the HTLV-1 infected/transformed cells (Yeung et al., 2008). In CD133(+) liver tumor-initiating cells, TP53INP1 is a direct target of miR-130b which promotes cell growth (Ma et al., 2010).

Tumor protein 53-induced nuclear protein 1 expression can also be modulated by miR-17-5p and miR-17. While miR-17-5p suppresses cell growth and promotes apoptosis of cervical cancer cells, TP53INP1 expression is reduced by miR-17-5p (Wei et al., 2012). In chronic lymphocytic leukemia (CLL) cells, up-regulation of miR-17~92 family miRNAs by Toll-like-receptor-9 agonists is preceded by a transient induction of the proto-oncogene Myc, and forced expression of miR-17, a major member from the miR-17~92 family, reduces TP53INP1 expression and protects cells against apoptosis (Bomben et al., 2012).

Additionally, TP53INP1 RNA can be targeted and down-regulated by miR-155 and miR-125b. miR-155 is overexpressed in pancreatic cancer cells and interacts with TP53INP1 mRNA at its 3'UTR (Gironella et al., 2007), whereas miR-125b is overexpressed in type II endometrial carcinoma cells and contributes to the

malignancy of type II endometrial carcinoma, possibly through down-regulation of TP53INP1 expression (Jiang et al., 2011). Regulation of TP53INP1 expression by miR-125b can be potentially important for more effective therapy, since various studies have established miR-125b as an oncogene or tumor suppressor gene in difference types of human tumors (Bousquet et al., 2010; Zhang et al., 2011; Bhattacharjya et al., 2013).

TP53INP1 INDUCES AUTOPHAGIC CELL DEATH

Autophagy is an important physiological response that is strongly induced during cellular stress, mainly under nutrient deficiency. Autophagy is basically a recycling event whereby the cellular organelles will be engulfed within the autophagosome and broken down upon contact with lysosome, consequently generating metabolites used for biosynthesis and energy metabolism to support the survival of cancer cells in the nutrient limited environment (Hanahan and Weinberg, 2011). Autophagy-related protein 5 (ATG5), beclin-1 and the light chain of the microtubule-associated protein 1 (LC3, a member of ATG8 family proteins) are key players in autophagic cell death (White, 2012).

While mainly a nuclear protein, TP53INP1 re-localizes into autophagosomes during autophagy, where TP53INP1 interacts with LC3 via a functional LC3-interacting region (Sancho et al., 2012; Seillier et al., 2012) (Figure 2). As TP53INP1 binds to LC3 with affinity higher than p62, the suppression of which promotes tumorigenesis (Mathew et al., 2009), TP53INP1 can partially displace p62 from autophagosomes and thus modify the composition of autophagosomes (Seillier et al., 2012).

Studies with mice with non-functional beclin-1 gene has shown increased susceptibility to cancer, due to the impairment in autophagy which requires to be circumvented during tumor development (Levine and Kroemer, 2008; White and DiPaola, 2009). Importantly, silencing beclin-1 or ATG5 or inhibiting caspase activity, which is necessary for the induction of autophagy, significantly decreases TP53INP1-induced cell death, indicating

the effect of TP53INP1 in inducing autophagic cell death (Sancho et al., 2012; Seillier et al., 2012).

TP53INP1 REPRESSES TUMOR CELL MIGRATION

One of the main features of cancer is the capability of cancer cells to migrate invasively through the stroma to form metastases, due to the significantly altered expression of the subset of genes involved in cell to cell and cell to extracellular matrix adhesion (Hanahan and Weinberg, 2011). TP53INP1 can work as a tumor suppressor by repressing tumor cell migration during metastasis. Secreted protein acidic and rich in cysteine (SPARC) regulates tumor cell-matrix interactions, and promotes cancer cell migration and metastasis (Podhajcer et al., 2008). SPARC gene expression is up-regulated in normal pancreas in the TP53INP1-deficient animals, and in pancreatic intraepithelial neoplasia lesions in a mouse model of pancreatic adenocarcinoma (Seux et al., 2011). TP53INP1 transcriptionally blocks SPARC gene expression, and silencing of TP53INP1 increases cell migration in mouse embryonic fibroblasts and pancreatic cancer cells (Seux et al., 2011). Consistent with these findings, miR-125b is overexpressed in type II endometrial carcinoma cells, and miR-125b expression increases endometrial carcinoma cell migration through down-regulating TP53INP1 expression (Jiang et al., 2011). Moreover, silencing TP53INP1 gene expression significantly correlates with lymphatic invasion in human gastric cancer patients (Jiang et al., 2006).

The role of TP53INP1 in suppressing cell migration is also of particular interest for cancer therapy, since the expression of genes involved in cell to cell and cell to extracellular matrix adhesion is significantly altered in some highly aggressive carcinomas (Hanahan and Weinberg, 2011), and TP53INP1 can potentially be an important player in invasion-metastasis cascade by targeting the genes. We propose that restoring TP53INP1 gene expression through targeting its silencers, such as miR-125, could effectively inhibit tumor invasion and metastasis.

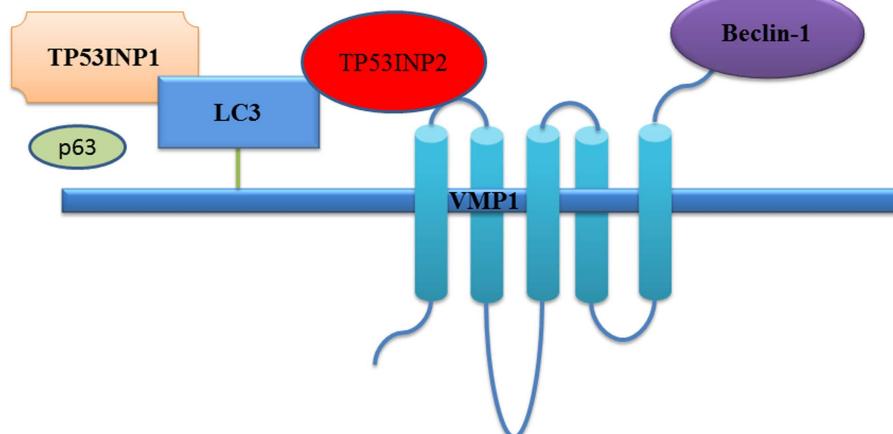


FIGURE 2 | TP53INP1 and TP53INP2 co-operatively induce autophagy.

Under autophagy-inducing stress such as starvation, TP53INP2 translocates from the nucleus to the autophagosomes, interacts with VMP1, and recruits

the critical autophagy regulator LC3 and beclin-1 (Nowak and Iovanna, 2009). TP53INP1 interacts with LC3 via a functional LC3-interacting region with affinity higher than p62, and displaces p62 from autophagosomes.

TP53INP1 EXPRESSION IS REDUCED IN HUMAN TUMOR TISSUES AND SILENCING OF TP53INP1 CONTRIBUTES TO TUMORIGENESIS *IN VIVO*

Tumor protein 53-induced nuclear protein 1 protein expression has been found to be present in non-malignant human pancreatic lesions, but significantly or completely lost in the majority of primary pancreatic ductal adenocarcinomas and absent in metastatic tumors (Gironella et al., 2007). Consistently, significant reduction in TP53INP1 expression has also been detected in human gastric (Jiang et al., 2006) and colon cancer tissues (Shibuya et al., 2010). These observations demonstrate that reduction in TP53INP1 expression might be a general feature of tumor development. As knocking-out TP53INP1 gene expression promotes, and forced overexpression of TP53INP1 reduces, pancreatic and liver tumorigenesis in mice (Gironella et al., 2007; Ma et al., 2010), the restoration of TP53INP1 expression could be an effective approach for cancer therapy.

TP53INP2 EXERTS EFFECTS DISTINCT FROM TP53INP1

Using a bioinformatics approach, Nowak et al. (2005, 2009) have identified TP53INP2, also known as diabetes and obesity regulated gene (DOR), as a TP53INP1-related gene. TP53INP2 encodes a protein with 30% amino acid identity and 45% similarity with TP53INP1, and shares two highly conserved regions (region 1: aa residues 28–42; region 2: 66–112 in human) with TP53INP1 (Sancho et al., 2012).

In spite of its homology with TP53INP1, TP53INP2 expression is not induced by p53, and forced overexpression of TP53INP2 does not alter the cell cycle or apoptosis. However, like TP53INP1, TP53INP2 is involved in the control of tumor development by modulating autophagy (Nowak et al., 2009). TP53INP2 functions as a scaffold protein, recruits LC3 and beclin-1 to the autophagosome through interacting with transmembrane protein vacuole membrane protein 1 (VMP1) (Nowak and Iovanna, 2009; Nowak et al., 2009), which is essential for autophagy (Ropolo et al., 2007). Upon autophagy-inducing stress such as starvation, TP53INP2 translocates from the nucleus to the autophagosomes, interacts with VMP1, recruits LC3, and beclin-1, but no beclin-2, and play an important role in autophagy (Nowak and Iovanna, 2009; Nowak et al., 2009) (Figure 2). Additionally, there are evidences suggesting that TP53INP1 and TP53INP2 can function as dual regulators of autophagy which make the proteins even more remarkable in controlling autophagy (Sancho et al., 2012).

TP53INP2 is also involved in tumor cell migration (Moran-Jones et al., 2009). Heterogenous nuclear ribonucleoprotein (hnRNPA2) is an important regulator of alternative splicing, is up-regulated in some invasive cancer types, and leads to tumor progression. It has recently been demonstrated that alternative splicing of exon 2 near the 5' un-translated region of TP53INP2 is a key event downstream of hnRNPA2 that is necessary for cancer cells to migrate and invade through the extracellular matrix (Moran-Jones et al., 2009).

Despite the important roles of TP53INP2 in cancer cell autophagy and migration, it is not clear how TP53INP2 expression is controlled in cancer cells. Additionally, it is important to

understand under which circumstances the splicing of TP53INP2 exon 2 by hnRNPA2 takes place, and whether hnRNPA2 also regulates alternative splicing of TP53INP1.

CONCLUSION

Tumor suppressive functions of p53 and its homolog p73 reflect physiological activities of a wide range of their target genes. The identification and functional characterization of the critical gene/genes responsible for p53 and p73 induced tumor suppressive functions are very important for understanding tumorigenesis and for designing better cancer therapy.

Tumor protein 53-induced nuclear protein 1 gene expression is often silenced in tumor cells due to oncogenic factors such as the micro RNAs miR-93, miR-130b, miR-155, miR-125b, miR-17-5p, and miR-17, which down-regulate TP53INP1 expression through post-transcriptional mechanisms. Upon exposure to genotoxic agents, p53 and p73 activates TP53INP1 gene expression by directly binding to the TP53INP1 gene promoter. Additionally, the transcription factor E2F1 directly up-regulates TP53INP1 gene transcription independent of p53 and p73, and the inflammatory mediators tumor necrosis factor α and interleukin 6 also enhance TP53INP1 gene expression.

The TP53INP1 gene encodes two protein isoforms, TP53INP1 α and TP53INP1 β . Upon initial DNA damage, p53 is phosphorylated at Ser-15 and Ser-20, stimulating the binding of p53 to promoter regions of a subset of genes such as the G1 arrest genes p21, the DNA repair gene p53R2, and the p53 negative regulators such as MDM2. If DNA damage is severe, TP53INP1 forms protein complexes with the protein kinases HIPK2 or PKC δ to phosphorylate p53 at Ser-46, promoting the binding of p53 to the promoter regions of apoptosis related genes such as p53AIP1 rather than the repair related genes, leading to cell growth arrest and apoptosis (Figure 1). Additionally, TP53INP1 facilitates p73-mediated apoptosis independent of p53, enhances autophagic cell death by interacting with LC3, and represses tumor cell migration via regulating SPARC expression. As TP53INP1 expression is frequently silenced or completely lost in human cancer tissues, restoration of TP53INP1 expression could potentially inhibit tumor growth via its anti-proliferative, pro-apoptotic, pro-autophagic, and anti-cell migration activities. While TP53INP1 expression can be indirectly up-regulated by chemicals which activate p53 expression, we anticipate that small molecule compounds which activate TP53INP1 gene promoter activity through binding to p53-binding sites could potentially restore TP53INP1 expression, and the small molecule compounds could be discovered through screening small molecule compound libraries.

The TP53INP1-related gene TP53INP2 has recently been identified. Unlike TP53INP1, TP53INP2 expression is not modulated by p53, and TP53INP2 does not modulate cell cycle progression and apoptosis. While TP53INP2 induces autophagy via mechanisms similar to TP53INP1, an alternative splicing product of TP53INP2 RNA due to hnRNPA2 induces cancer cell migration. It is crucial to further understand how to control hnRNPA2-mediated TP53INP2 splicing to avoid cancer cell migration,

and to retain TP53INP2-mediated autophagy, which synergizes with TP53INP1-induced autophagy. As such, restoration of TP63INP1 and TP53INP2 expression without the induction of hnRNPA2-mediated TP53INP1 RNA alternative splicing would ideally induce cancer cell cycle arrest, apoptosis, and autophagy, therefore simultaneously inducing binary cell death for a more effective therapy.

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On the road with WRAP53 β : guardian of Cajal bodies and genome integrity

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The *WRAP53* gene encodes both an antisense transcript (*WRAP53* α) that stabilizes the tumor suppressor p53 and a protein (*WRAP53* β) involved in maintenance of Cajal bodies, telomere elongation and DNA repair. *WRAP53* β is one of many proteins containing WD40 domains, known to mediate a variety of cellular processes. These proteins lack enzymatic activity, acting instead as platforms for the assembly of large complexes of proteins and RNAs thus facilitating their interactions. *WRAP53* β mediates site-specific interactions between Cajal body factors and DNA repair proteins. Moreover, dysfunction of this protein has been linked to premature aging, cancer and neurodegeneration. Here we summarize the current state of knowledge concerning the multifaceted roles of *WRAP53* β in intracellular trafficking, formation of the Cajal body, DNA repair and maintenance of genomic integrity and discuss potential crosstalk between these processes.

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Introduction

The eukaryotic cell nucleus is highly organized with several sub-compartments containing high concentrations of factors involved in specific biological processes to optimize performance. Numerous distinct, non-membrane-bound nuclear bodies, including nucleoli, nuclear speckles, histone locus bodies, promyelocytic leukemia (PML) bodies and Cajal bodies, have been identified and shown to overlap with respect to their components and organization. Formation of such sub-organelles usually involves dynamic processes, such as protein modifications and self-association, various RNA-protein interactions and tethering of central factors to specific gene loci (Dundr and Misteli, 2010; Machyna et al., 2013).

Identified more than a century ago and subjected to intense functional investigation, the Cajal body has been suggested to be associated with telomere maintenance and maturation of the splicing machinery (Machyna et al., 2013). In a similar manner, DNA repair factors are sequestered in specialized repair centers called foci. Following induction of DNA double-strand breaks, a variety of proteins are mobilized to the break sites to initiate the signaling cascades required for proper repair (Polo and Jackson, 2011).

WRAP53 β (also denoted WRAP53 or WDR79 or TCAB1), is a scaffold protein that directs factors to Cajal bodies, telomeres and DNA double-strand breaks, thereby facilitating the interactions necessary for appropriate biological responses (Tycowski et al., 2009; Venteicher et al., 2009; Mahmoudi et al., 2010).

The WD40 domain of WRAP53 β appears to be critical for its function, by serving as a scaffold for multiple interactions between a wide variety of molecules. Mutations predicted to impair the structure of this domain cause dyskeratosis congenita, a syndrome associated with premature aging and an elevated predisposition for cancer, highlighting the importance of WRAP53 β for homeostasis (Zhong et al., 2011).

In the present review, we focus on WRAP53 β and its reported roles in the maintenance of the Cajal body, as a component of the telomerase enzyme and, recently, in DNA damage response and repair. We also discuss the involvement of this protein in various diseases.

WRAP53: One Gene—Multiple Products

As its name indicates, the WD40-encoding RNA antisense to p53 (*WRAP53*) gene, identified in our laboratory, encodes at least two different functional products: an antisense transcript that stabilizes p53 (referred to as WRAP53 α) and, via alternative transcriptional start site usage, a protein containing WD40 repeats (referred to as WRAP53 β , alias WRAP53, WDR79 and TCAB1). Moreover, a third alternative start site in exon 1 γ gives rise to WRAP53 γ transcripts overlapping the first intron of p53, the function of which remains elusive and is not discussed further here (Figure 1) (Farnebo, 2009; Mahmoudi et al., 2009; Tycowski et al., 2009; Venteicher et al., 2009).

WRAP53 α : a Natural p53 Antisense Transcript

The *WRAP53* gene on chromosome 17p13 partially overlaps the *p53* tumor suppressor gene in a head-to-head orientation

(Figure 1). This organization has functional consequences, i.e., WRAP53 α transcripts containing this overlap regulate the levels of p53 mRNA and protein.

By binding to the 5' UTR region of p53 mRNA through a perfectly complementary sequence, WRAP53 α stabilizes this mRNA, thereby promoting induction of the p53 protein in response to DNA damage required for mediating apoptosis (Mahmoudi et al., 2009). The insulator protein CTCF contributes to WRAP53 α -mediated regulation of p53 by binding WRAP53 α RNA (Saldana-Meyer et al., 2014). Several lines of evidence indicate that neither WRAP53 β transcripts nor protein are involved in regulating p53: (1) overexpression of the overlapping exon 1 α , which is not present in WRAP53 β transcripts, efficiently elevates the steady-state level of p53; whereas overexpression of exon 1 β or the WRAP53 β protein has no such effect. (2) siRNAs targeting exon 1 α , but not exon 1 β down-regulate p53. And (3) knock-down of WRAP53 α transcripts does not alter WRAP53 β protein levels, but nevertheless reduces p53 expression (Farnebo, 2009; Mahmoudi et al., 2009).

WRAP53 β : an Essential Cajal Body Component

Most investigations on the *WRAP53* gene have focused on the WRAP53 β (WRAP53/WDR79/TCAB1) protein, which is highly evolutionary conserved, with homologs (confined to its WD40 repeats) in vertebrates, invertebrates, plants and yeast. A WD40 repeat is a short structural motif containing approximately 40 amino acids and often a C-terminal tryptophan (W) and aspartic acid (D) dipeptide. Typically multiple WD40 repeats exist within a WD40 domain, which allow interaction with several partners simultaneously, in a non-exclusive manner (Stirnimann et al., 2010; Xu and Min, 2011).

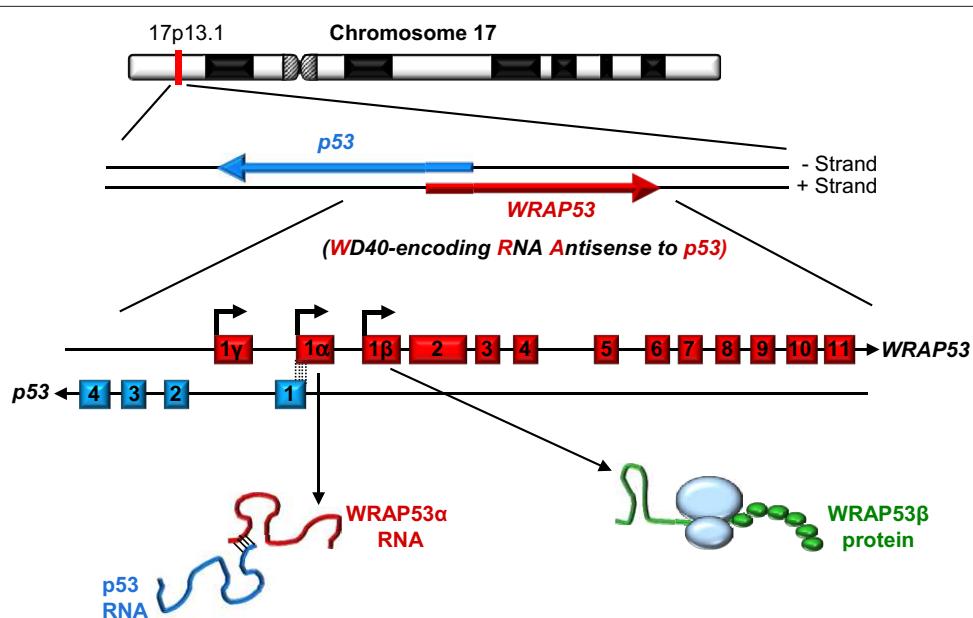


FIGURE 1 | The *WRAP53* and *p53* genes and their gene products. The arrows indicate the direction of transcription and the dotted lines the exon overlap between these genes.

WRAP53 β consist of a proline-rich N-terminus (16 of 50 residues = 32%), a central WD40 domain (predicted to contain 5–7 repeats) and a glycine-rich C-terminus (7 of 13 residues = 54%) (**Figure 2A**).

The WRAP53 β protein is found both in the cytoplasm and highly enriched in nuclear organelles known as Cajal bodies (**Figure 2B**) (Mahmoudi et al., 2010). These spherical organelles (0.2–2 μ M), were identified and described by Santiago Ramón y Cajal in 1903 (Gall, 2003). Their numbers range from 1 to 10 per nucleus, being highest in cells with rapid rates of transcription and splicing (Cioce and Lamond, 2005; Boulon et al., 2010).

Cajal bodies are characterized by the marker protein coilin that interacts with many factors and thereby serves as a platform for the assembly of Cajal bodies. Cajal bodies are rich in ribonucleoprotein (RNP) complexes, including the spliceosomal small nuclear RNPs (snRNPs), small Cajal body-specific RNPs (scaRNPs), small nucleolar RNPs (snoRNPs) and components of the telomerase RNP complex. Accordingly, Cajal bodies have been described to play essential roles in the maturation of snRNPs and snoRNPs and telomere maintenance. Furthermore, the survival of motor neuron (SMN) complex and factors involved in 3'-end processing of histone mRNA accumulate in these bodies (Carvalho et al., 1999; Machyna et al., 2013). Defects in Cajal body formation are linked to impaired cell proliferation and splicing, but rather than being essential for the associated processes, this compartment is thought to enhance efficiency by concentrating necessary factors in the same space (Lemm et al., 2006; Whittom et al., 2008; Novotny et al., 2011).

Cajal bodies are highly dynamic, moving within the nucleoplasm except when transiently immobilized through interaction with chromatin. These interactions occur at specific loci with repeated clusters of snRNA, U3 snoRNA and histone genes and in a transcription-dependent manner (Frey and Matera, 1995; Gao et al., 1997; Frey et al., 1999; Platani et al., 2000). Tethering experiments have revealed that immobilization of various constituents leads to the de novo formation of Cajal bodies, a self-organized process that appears to occur in random order, without internal hierarchy of individual components (Kaiser et al., 2008; Shevtsov and Dundr, 2011).

Several studies have emphasized the role of WRAP53 β as a central player in maintenance of and localization of factors to the Cajal body (**Figure 2C**). Indeed, without WRAP53 β these organelles collapse and cannot re-form. Exogenous WRAP53 β accumulates in Cajal bodies, but does not stimulate *de novo* formation of this organelle. Instead, Cajal bodies are disrupted when high levels of this protein are exogenously expressed, indicating an adverse effect on the function of endogenous WRAP53 β , potentially via self-association or sequestration of certain factors important for Cajal body formation (Mahmoudi et al., 2010). Similar effects have been observed when coilin is exogenously overexpressed (Hebert and Matera, 2000).

In addition to maintaining their structural integrity, WRAP53 β targets several factors to Cajal bodies and loss of this trafficking is associated with various disorders (see further below). Depletion of this protein causes many factors to mislocalize to the nucleolus (Mahmoudi et al., 2010). This may indicate that WRAP53 β promotes the translocation of

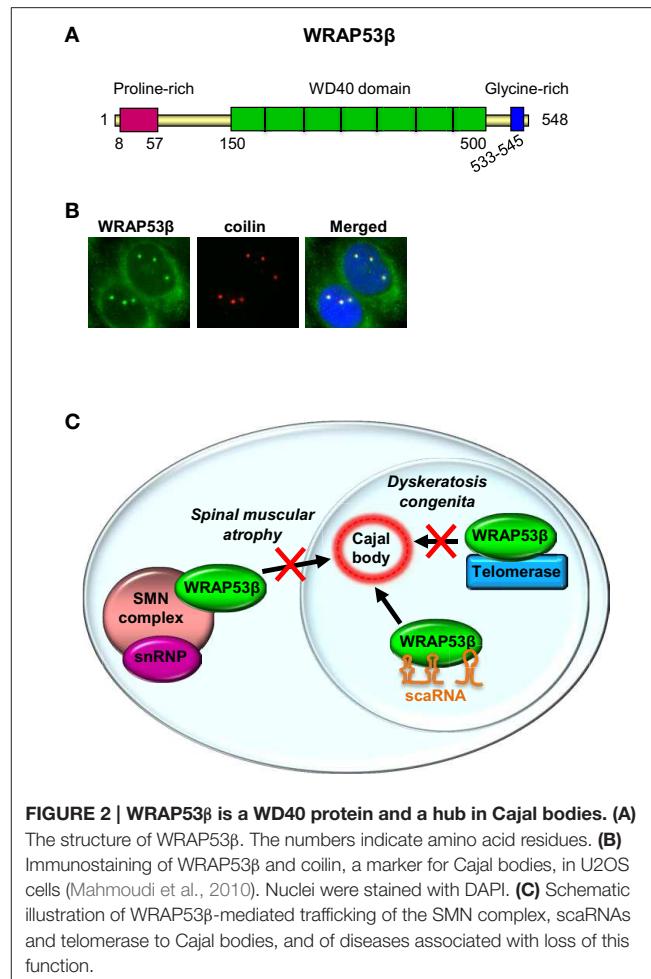


FIGURE 2 | WRAP53 β is a WD40 protein and a hub in Cajal bodies. **(A)** The structure of WRAP53 β . The numbers indicate amino acid residues. **(B)** Immunostaining of WRAP53 β and coilin, a marker for Cajal bodies, in U2OS cells (Mahmoudi et al., 2010). Nuclei were stained with DAPI. **(C)** Schematic illustration of WRAP53 β -mediated trafficking of the SMN complex, scaRNAs and telomerase to Cajal bodies, and of diseases associated with loss of this function.

factors from the nucleolus to Cajal bodies or alternatively by binding and directing factors to these bodies, prevents them from accumulating in nucleoli. WRAP53 β does not regulate localization of factors to other nuclear organelles, such as gems, splicing speckles and PML bodies (Mahmoudi et al., 2010).

WRAP53 β Guides SMN Across the Cell to Cajal Bodies

One factor, which WRAP53 β helps to localize to Cajal bodies, is the SMN protein that together with its partner proteins Gemin2-8 and Unrip, forms the SMN complex involved in the cytoplasmic assembly of the spliceosomal snRNPs (Gubitz et al., 2004; Chari et al., 2008; Cauchi, 2010; Matera and Wang, 2014). By binding Sm proteins and snRNAs separately, the SMN complex promotes Sm binding specifically to snRNA (Pellizzoni et al., 2002).

WRAP53 β is involved in the translocation of SMN across the cell. After binding SMN in the cytoplasm, WRAP53 β first recruits this protein to the nucleus by promoting its interaction with the nuclear pore receptor importin β and then targets SMN to Cajal bodies by facilitating its interaction with coilin. Thus, knockdown of WRAP53 β results in accumulation of SMN in the cytoplasm

and mislocalization of nuclear SMN to the nucleolus. WRAP53 β itself is targeted to Cajal bodies via its WD40 domain and a stretch of its C-terminus, and these same regions appears to scaffold the SMN-coilin interaction (Mahmoudi et al., 2010).

Is the WRAP53 β Route Blocked in Spinal Muscular Atrophy?

Mutations in the *SMN1* gene results in spinal muscular atrophy (SMA), a neurodegenerative disorder characterized by progressive degeneration of spinal cord anterior horn α -motor neurons and the leading genetic cause of infant mortality with an incidence of approximately 1:6000 live births (Coady and Lorson, 2011). The reason why spinal motor neurons are particularly sensitive to mutations of the SMN protein is not fully understood. Cells lacking SMN display impaired assembly of the snRNP core, along with splicing defects (Fischer et al., 1997; Pellizzoni et al., 2002; Zhang et al., 2008), however, the fact that most human cells require splicing points to additional functions of SMN important for this cell type. In line with such an idea, the nuclear function of SMN remains unclear and the severity of the SMA disease increases as the level of SMN protein and number of SMN-containing nuclear structures decreases (Lefebvre et al., 1997; Oskoui et al., 2007; Tapia et al., 2012).

Interestingly, defective WRAP53 β -mediated trafficking of SMN is observed in patients afflicted by the most severe form of spinal muscular atrophy (type I or Werdnig-Hoffmann disease) (Lefebvre et al., 1997; Oskoui et al., 2007; Mahmoudi et al., 2010; Tapia et al., 2012). This impaired interaction could not be explained by the lower amount of SMN protein present; instead, WRAP53 β can apparently not bind properly to SMN and localize SMN to Cajal bodies in these cells (Mahmoudi et al., 2010). Since WRAP53 β recently was identified as an important regulator of DNA double-strand break repair (Henriksson et al., 2014), it is possible that SMN collaborates with WRAP53 β in this process and that impaired DNA repair contributes to the pathogenesis of SMA.

Another Highway: WRAP53 β Targets ScaRNAs to Cajal Bodies

WRAP53 β also guides a class of RNA molecules referred to as scaRNAs to Cajal bodies, which are required for catalyzing post-transcriptional modifications (including pseudouridylation and 2'-O-methylation) of the snRNA component of snRNPs (Tycowski et al., 2009). These modifications are important for their proper incorporation into the spliceosome (Darzacq et al., 2002; Jady et al., 2003).

The scaRNA family consists of at least 20 members, including the RNA component of telomerase (TERC). They are divided into two major classes: the C/D box scaRNAs, which contain the C (RUGAUGA) and D (CUGA) motifs and direct methylation of target snRNAs, and the H/ACA box scaRNAs, which contain the H (ANANNA) and ACA motifs and guide isomerization of uridine into pseudouridine. The C/D box scaRNAs associate with four core proteins: the methyltransferase fibrillarin, NOP56,

NOP58 and 15.5K/NHPX, while the H/ACA box scaRNAs associate with four other core proteins: the pseudouridine synthase dyskerin, GAR1, NHP2, and NOP10. The RNA components of the scaRNPs direct the enzymes and associated proteins to their target RNAs via sequence complementarity (Kiss, 2002; Kiss et al., 2006).

The scaRNAs are targeted to Cajal bodies by a common element, referred to as the Cajal body localization signal or CAB box. The consensus sequence of this element is: ugAG for H/ACAs scaRNA and GU-rich dinucleotide repeats in the case of C/D box scaRNAs (Richard et al., 2003; Tycowski et al., 2009; Marnef et al., 2014). Although, the factor(s) responsible for this targeting long remained unknown, WRAP53 β has now been shown to associate specifically with the CAB box of scaRNAs and promote their targeting to Cajal bodies. Thus, CAB box mutations that disturb binding to WRAP53 β or depletion of this protein results in mislocalization of scaRNAs to nucleoli (Tycowski et al., 2009).

Although WRAP53 β does not appear to bind snRNAs or snoRNAs that lack CAB box motifs it does bind to another class of RNAs, the AluACA RNAs (Jady et al., 2012). Interestingly, these RNAs originate from Alu repeats, are processed into RNA containing H/ACA and CAB boxes and also associate with dyskerin, NOP10, NHP2 and GAR1. Despite their CAB box motifs, the AluACA RNAs accumulate in the nucleoplasm rather than Cajal bodies and their function is not known.

WRAP53 β : the Telomerase Taxi

The telomerase RNP holoenzyme catalyzes the addition of telomeric repeats TTAGGG onto the ends of linear chromosomes. The minimal catalytic unit consists of a reverse transcriptase (TERT) and the TER/C RNA template containing the sequence copied by TERT (Artandi and Depinho, 2010). WRAP53 β associates with the TER/C CAB box of the enzymatically active telomerase complex and promotes its localization to Cajal bodies (Venteicher et al., 2009). Since TER/C is an H/ACA scaRNA, it also binds the scaRNP core proteins dyskerin, GAR1, NHP2 and NOP10 that play important roles in the stability, nuclear localization and proper assembly of telomerase RNP (Artandi and Depinho, 2010). Structural deviations and a lack of complementary target RNAs indicate that TER/C is not directly involved in pseudouridylation of snRNAs (Mitchell et al., 1999; Trahan and Dragon, 2009; Egan and Collins, 2012).

The observation that WRAP53 β binds all core components of telomerase (including TER/C, TERT and dyskerin), but not to telomerase assembly factors (including NAF1, pontin and reptin), indicates that this protein is a component of the active telomerase enzyme. WRAP53 β is required for telomerase localization to Cajal bodies, which associate with telomeres during S-phase (Jady et al., 2006; Tomlinson et al., 2006; Venteicher et al., 2009). Knockdown of WRAP53 β disrupts targeting of TER/C to both Cajal bodies and telomeres and, consequently, leads to progressive telomere shortening. Similarly, cells with TER/C containing a CAB box mutation also display telomere shortening, probably due to mislocalization of TER/C to the nucleolus (Venteicher et al., 2009; Egan and Collins, 2010). However, telomerase function and telomere elongation was recently shown

to be unaffected in Cajal body-deficient coolin knockout cells. This suggest that Cajal bodies not are essential in this process, whereas certain Cajal body factors such as WRAP53 β clearly play important roles in telomere homeostasis. Additional studies are required to clarify the previous suggested role for the Cajal body in telomere biology (Chen et al., 2015).

WRAP53 β and Dyskeratosis Congenita

Germline mutations in WRAP53 β result in dyskeratosis congenita, a syndrome characterized by bone marrow failure, premature aging, predisposition for cancer and a triad of mucocutaneous features including oral leukoplakia, abnormal skin pigmentation and nail dystrophy (Zhong et al., 2011). This disease is caused by defective telomere maintenance, since approximately 50% of all cases carry mutations in core components of the telomerase enzyme or in telomere capping proteins, such as TERC, TERT, dyskerin, TIN2 and WRAP53 β (Dokal, 2011; Zhong et al., 2011; Ballew and Savage, 2013).

Although patients with dyskeratosis congenita display very short telomeres, clinical characteristics, such as age at onset and disease severity are not strictly correlated to telomere length. Moreover, with certain associated TERT mutations telomerase activity is maintained (Vulliamy et al., 2011; Zaug et al., 2013). Therefore, additional perturbations, such as impaired stem cell function and defects in rRNA processing and DNA repair, might be involved in the etiology of dyskeratosis congenita (Ruggiero et al., 2003; Mochizuki et al., 2004; Zhang et al., 2012b; Bellodi et al., 2013).

Mutations in WRAP53 β are inherited in an autosomal recessive fashion and reside in highly conserved regions of its WD40 domain. These mutations reduce the nuclear level of WRAP53 β , impair its trafficking of telomerase to telomeres, and subsequently lead to progressive shortening of telomeres in these patients (Zhong et al., 2011). Recently, the chaperonin CCT/TRiC was identified to be crucial for proper folding of WRAP53 β and this folding was found to be impaired in dyskeratosis congenita (Freund et al., 2014). Since mutated and unfolded WRAP53 β is not translocated into the nucleus, all of its activities in this organelle should be disturbed.

Interestingly, our group demonstrated recently that WRAP53 β is involved in the repair of DNA double-strand breaks (Henriksson et al., 2014), which thus might contribute to disease onset and severity in patients with dyskeratosis congenita. Since this function is independent of telomerase activity, the clinical differences between patients with WRAP53 β or TERT/TERC mutations might be due to accumulation of DNA damage. Indeed, mutations in WRAP53 β result in a more severe form of this disease (Dokal, 2011; Ballew and Savage, 2013). Similarly, mutations in dyskerin cause a severe variant of dyskeratosis congenita with elevated numbers of γ H2AX foci in response to induction of DNA double-strand breaks. The majority of these foci were not localized to telomeres, suggesting a general enhancement in DNA damage (Gu et al., 2008).

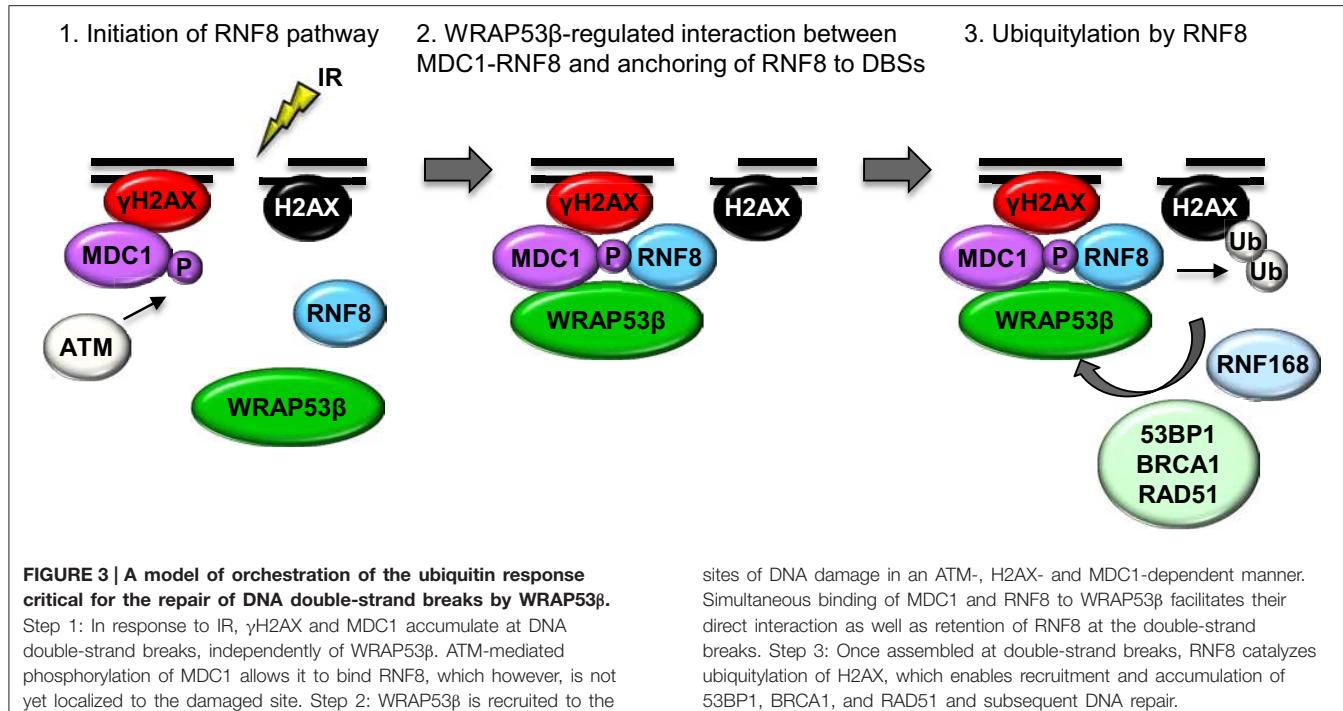
WRAP53 β Takes a New Route—to DNA Double-Strand Breaks

Among the most cytotoxic DNA lesions are double-strand breaks, which are repaired by the homologous recombination (HR) or non-homologous end joining (NHEJ) pathways and involves stepwise accumulation of repair proteins at the site of damage. One of the earliest events following DNA double-strand breakage is phosphorylation of the nearby H2AX histone variant on serine-139 (to form γ H2AX) catalyzed by ATM, ATR, and DNA-PK kinases (Durocher and Jackson, 2001). Next, the adaptor protein MDC1 binds γ H2AX via its tandem BRCT domain and is subsequently phosphorylated by ATM. MDC1 serves as an anchor for the assembly of a variety of proteins to the site of DNA damage (Stucki et al., 2005), including RNF8, which is the first E3 ligase to be recruited to these breaks. The FHA domain of RNF8 binds to ATM-phosphorylated residues of MDC1 (four TQXF clusters). Via its RING domain, RNF8 then ubiquitylates histones H2A and H2AX at DNA damages sites, which in turn promotes accumulation of downstream factors (Huen et al., 2007; Kolas et al., 2007; Mailand et al., 2007).

The first indication that WRAP53 β is involved in the repair of DNA double-strand breaks was its accumulation at such breaks induced by either whole cell or laser micro-irradiation. In a manner dependent on the DNA damage response protein kinase ATM, histone H2AX and MDC1, WRAP53 β is rapidly recruited to DNA breaks, reaching maximal levels 1 h after damage and thereafter gradually declining as the DNA breaks are repaired. Loss of WRAP53 β leads to prolonged cell cycle arrest at the G2/M stage following irradiation, as well as more spontaneous DNA breaks, which are considered to be the major cause of genomic instability (Henriksson et al., 2014). Moreover, in cells depleted of WRAP53 β recruitment of repair factors to DNA breaks is impaired and both the HR and NHEJ repair pathways are defective (Henriksson et al., 2014). Mechanistically, WRAP53 β target the critical ubiquitin ligase RNF8 to DNA lesions by mediating the interaction with its upstream partner MDC1. WRAP53 β binds the FHA domains of both RNF8 and MDC1 simultaneously via its WD40 domain, thereby facilitating accumulation of RNF8 and ubiquitylation at DNA double-strand breaks (Henriksson et al., 2014). Accordingly, WRAP53 β is required for the assembly of downstream repair proteins such as 53BP1, BRCA1, and RAD51 at DNA breaks (Figure 3) (Henriksson et al., 2014).

The Two Faces of WRAP53 β in Cancer

One of the effects of WRAP53 β depletion observed earliest was apoptosis of cancer cells *in vitro* whereas normal cells were unaffected (Mahmoudi et al., 2011). The subsequent finding that WRAP53 β is overexpressed in a variety of cancer cell lines of different origins and that such overexpression promotes carcinogenic transformation indicated that this protein possesses oncogenic properties (Mahmoudi et al., 2011). In agreement with this proposal, WRAP53 β has been found to be overexpressed in primary nasopharyngeal carcinoma (Sun et al., 2014), esophageal squamous cell carcinoma (Rao et al., 2014) and rectal cancer



(Zhang et al., 2012a). Moreover, knockdown of WRAP53 β in cancer cells reduced the size of the tumors formed when these are grafted into mice (Sun et al., 2014).

The role of WRAP53 β in telomere elongation can at least partially explain its oncogenic properties, since re-activation of telomerase is what immortalizes 90% of all human cancer (Kyo and Inoue, 2002). At the same time, knockdown of WRAP53 β in cancer cells induces apoptosis within 48–72 h, whereas knockdown of telomerase enhances cell death only after several weeks in culture (Shammas et al., 2005). The observations that inactivating mutations in both alleles of WRAP53 β causes dyskeratosis congenita, indicates that this protein acts as tumor suppressor, rather than an oncogene.

Indeed, with its complex roles in a number of cellular processes, WRAP53 β may act as a tumor suppressor under certain conditions and as an oncogene in others. The subcellular localization of WRAP53 β may also explain some of its contradictory effects in tumor cells. For example, loss of nuclear, but not cytoplasmic WRAP53 β is correlated survival and resistance to radiotherapy in patients with head and neck cancer (Garvin et al., 2015). Thus, the levels of this protein in the nucleus and cytoplasm must be considered separately in connection with patient prognosis.

Single nucleotide polymorphisms (SNPs) in the WRAP53 gene have been linked to an increased risk for breast and ovarian cancer (Garcia-Closas et al., 2007; Schildkraut et al., 2009; Medrek et al., 2013). One of these SNPs is also associated with defective DNA repair and hematotoxicity in workers exposed to benzene. SNPs in WRAP53 and four other genes (*BLM*, *WRN*, *RAD51*, and *TP53*), the products of which play key roles in DNA repair and maintenance of genomic integrity, especially via the HR pathway

(Lan et al., 2009), have been found to predispose to benzene toxicity. At this point, it is unclear how the SNPs in WRAP53 alter the functions of the WRAP53 β protein.

In conclusion, WRAP53 β dysfunction is associated with many diseases, but it is currently unknown whether accumulation of DNA damage and/or deficient DNA repair contributes to their etiology.

WRAP53 β Unwraps the Crosstalk between Cajal Bodies, RNA Processing, Telomeres and the DNA Damage Response

DNA damaging agents, such as UV, cisplatin and IR all disrupt Cajal bodies and results in mislocalization of the marker protein coolin to microfoci and nucleoli. Moreover, coolin depletion enhances cell viability upon cisplatin treatment (Ciocca et al., 2006; Boulon et al., 2010; Gilder et al., 2011). Such observations clearly reveal that the Cajal body responds to stress.

Several components of the Cajal body, including WRAP53 β , have been linked to the DNA damage response. Coolin interacts with Ku70/Ku80 and inhibits NHEJ, presumably by preventing recruitment of the Ku proteins to DNA ends (Velma et al., 2010). SMN and Gemin2, members of the SMN complex, promote RAD51 assembly at DNA double-strand breaks and HR (Takaku et al., 2011). Interestingly, SMN interacts with methylated H3K79 in chromatin via its Tudor domain, a site known to target 53BP1 to DNA double-strand breaks (Huyen et al., 2004; Sabra et al., 2013). Notably, coolin also contains a Tudor domain that similar to SMN binds demethylarginine, as shown for Sm proteins (Tripsianes et al., 2011; Tapia et al., 2014).

Certain factors involved in the DNA damage response are localized in Cajal bodies. For instance in *Drosophila*, following auto-modification, PARP translocates from chromatin to Cajal bodies (Kotova et al., 2009) and WRAP53 β might also shuttle between these same two compartments. Furthermore, the SUMO E3 ligase PIAS4 (also known as PIASy), which accumulates at DNA double-strand breaks and is required for efficient RNF8-mediated ubiquitylation at sites of DNA damage, is present in the Cajal body (Sun et al., 2005; Galanty et al., 2009). A number of large-scale screens for factors involved in the DNA damage response have revealed an enrichment of proteins involved in RNA processing, although their exact involvement is not yet understood (Li and Manley, 2005; Montecucco and Biamonti, 2013).

Only future investigations can reveal whether specific components of the Cajal body contribute to DNA repair, including repair events with which WRAP53 β is associated. Moreover, the impact of the RNA-related activities of WRAP53 β on the DNA damage response and/or the phenotypes associated with a deficiency in this protein remains to be determined. It is also unknown whether cells of patients with spinal muscular atrophy exhibit elevated DNA damage or deficient DNA repair. In any case, since coilin depletion does not disrupt DNA repair (Henriksson et al., 2014), maintenance of the structure of Cajal body is not linked directly to this process.

Telomeres represent an important additional link between WRAP53 β , Cajal bodies and the DNA damage response. DNA repair proteins are present on both functional and dysfunctional telomeres. In the case of functional telomeres, these factors promote homeostasis and prevent end-joining events. For example, DNA-PKcs appear to promote telomere capping, thereby attenuating telomere fusion (D'adda Di Fagagna et al., 2004). Moreover, Ku70/80 interacts directly with TERC to promote telomere maintenance (Ting et al., 2005). Dysfunctional, uncapped telomeres are recognized as DNA double-strand breaks by the DNA damage response, resulting in the assembly of repair factors into local telomere dysfunction-induced foci (TIFs) (Takai et al., 2003). HR and NHEJ at such sites gives rise to chromosome fusions and genomic instability, demonstrating that DNA repair at the wrong place can result in genomic instability. For example, RNF8 promotes the assembly of repair proteins at telomeres by ubiquitylating their ends, thereby facilitating chromosome fusion in cases of telomere dysfunction (Peuscher and Jacobs, 2011). Furthermore, 53BP1 increases the mobility of dysfunctional telomeres, bringing chromosomal ends into close proximity and thereby allowing NHEJ (Dimitrova et al., 2008). The findings that WRAP53 β is involved in the recruitment of both RNF8/53BP1 to DNA double-strand breaks and telomerase to telomeres raises the interesting possibility that WRAP53 β regulate these processes via a common mechanism.

Telomerase-deficient ALT cells are characterized by very long and heterogeneous telomeres maintained by HR. In analogy to the role of Cajal bodies in telomerase-dependent telomere elongation (Jady et al., 2006; Tomlinson et al., 2006), a specific subpopulation of telomere-associated PML bodies has been proposed to promote the recombination in ALT cells. In addition to the conventional components of the PML body, these

ALT-associated PML bodies contain many proteins involved in the DNA damage response (Brault and Autexier, 2011). NBS1 is essential for their assembly: depletion of this protein leads to smaller number of ALT-associated PML bodies and telomere shortening in ALT-cells, but has no such effect on telomerase-positive cells (Wu et al., 2003; Zhong et al., 2007). Such observations indicate an intriguing link between nuclear body compartmentalization, telomere elongation and DNA damage response proteins.

Concluding Remarks and Future Perspectives

All of the functions described for WRAP53 β involve the recruitment and proper targeting of factors to specific cellular sites (**Figure 4**). This protein binds the SMN complex in the cytoplasm and subsequently promotes its entry into the nucleus and localization to Cajal bodies. Patients with spinal muscular atrophy exhibit defective binding of WRAP53 β to SMN, as well as reduced accumulation of SMN in Cajal bodies (Mahmoudi et al., 2010). Moreover, WRAP53 β regulates telomerase localization to Cajal bodies, as well as to telomeres, and disruption of this trafficking causes dyskeratosis congenita (Venteicher et al., 2009; Zhong et al., 2011).

In addition, WRAP53 β binds scaRNAs and promotes their localization to Cajal bodies (Tycowski et al., 2009). Finally, this protein binds the E3 ligase RNF8, facilitating its interaction with MDC1, which is required for its proper localization to DNA breaks and downstream repair events (Henriksson et al., 2014). Clearly, WRAP53 β is as an essential scaffold protein that interacts with many types of RNA and protein, contributing both to their intracellular trafficking and interaction with other factors. However, it remains to be determined whether other Cajal body components also play a role in DNA repair, including repair associated with WRAP53 β .

The discovery that inherited mutations in WRAP53 causes the syndrome dyskeratosis congenita, which predisposes

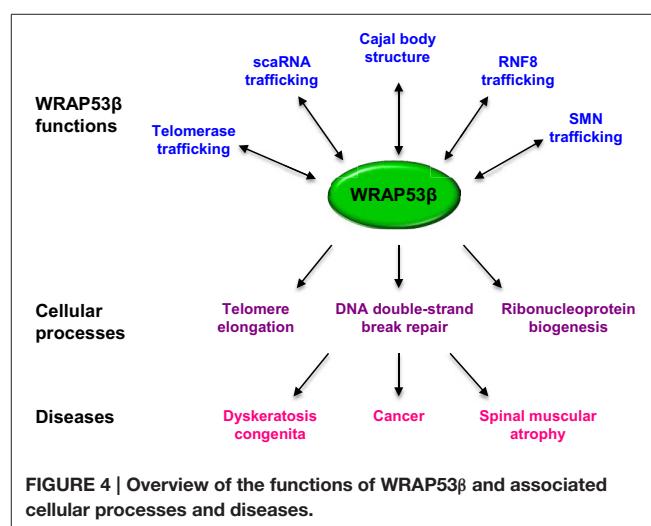


FIGURE 4 | Overview of the functions of WRAP53 β and associated cellular processes and diseases.

for cancer, suggests that this is a tumor suppressor gene. However, WRAP53 β also possesses oncogenic properties and can be a potential target of cancer therapy (Mahmoudi et al., 2011). Disruption of Cajal bodies is expected to decrease production of mature snRNPs, resulting in inefficient splicing, and inhibitors of the spliceosome have shown anti-tumor activities (Van Alphen et al., 2009). In addition, deficient WRAP53 β -mediated trafficking of telomerase results in telomere shortening, both *in vitro* and *in vivo* (Venteicher et al., 2009). Moreover, the pathways involved in DNA repair are also targets for cancer therapy, either directly or in combination with DNA-damaging agents (Helleday et al., 2008). Further insights into the physiological roles of WRAP53 β

and its contribution to the development of cancer might be provided by transgenic animal models.

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The DNA damage checkpoint response to replication stress: A Game of Forks

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Conditions challenging replication fork progression, collectively referred to as replication stress, represent a major source of genomic instability and are associated to cancer onset. The replication checkpoint, a specialized branch of the DNA damage checkpoint, monitors fork problems, and triggers a cellular response aimed at preserving genome integrity. Here, we review the mechanisms by which the replication checkpoint monitors and responds to replication stress, focusing on the checkpoint-mediated pathways contributing to protect replication fork integrity. We discuss how cells achieve checkpoint signaling inactivation once replication stress is overcome and how a failure to timely revert checkpoint-mediated changes in cellular physiology might impact on replication dynamics and genome integrity. We also highlight the checkpoint function as an anti-cancer barrier preventing cells malignant transformation following oncogene-induced replication stress.

Keywords: replication forks, DNA damage checkpoint, genomic instability, Mec1/ATR, oncogene stress

INTRODUCTION

During S phase, cells must faithfully duplicate their genomes. For this purpose eukaryotic cells establish multiple replication forks, specialized structures where DNA synthesis is carried out, that traverse the entire genome in a coordinated manner, thus granting a timely chromosomal replication (Bell and Dutta, 2002). Replication forks are complex structures in which parental DNA is unwound to produce a single-stranded DNA (ssDNA) template for replicative DNA polymerases (Johnson and O'Donnell, 2005). Due to the presence of ssDNA and the necessity to finely tune the functions of the diverse replisome components, replication forks are fragile structures prone to accumulating DNA breaks and being engaged by recombinational repair machineries (Branzei and Foiani, 2010). Conditions that impair DNA synthesis at replication forks or interfere with their progression, collectively termed "replication stress", can alter replication fork structure and functionality thus priming chromosomal breakage and unscheduled recombination events. Recent evidence has suggested that replication stress can be a major source of spontaneous genomic instability driving malignant transformation of pre-cancerous cells (Bartek et al., 2007b).

Eukaryotic cells have evolved mechanisms, usually termed as the replication checkpoint, that monitor the occurrence of replication stress and trigger a cellular response aimed at preserving genome integrity. The replication checkpoint constitutes a specialized branch of the DNA damage checkpoint and it is often referred to as the S phase (or intra-S phase) checkpoint (Paulovich and Hartwell, 1995; Boddy and Russell, 2001; Nyberg et al., 2002; Osborn et al., 2002). Even if it was originally described as a signal transduction pathway delaying cell

cycle progression to provide time to allow replication to finish (Enoch and Nurse, 1990; al-Khodairy and Carr, 1992; Enoch et al., 1992; Rowley et al., 1992; Weinert, 1992), work over the last 25 years has revealed that the replication checkpoint is a complex response with highly interconnected players, which regulates an unprecedented variety of cellular processes in order to sustain cell viability and protect genome integrity (Branzei and Foiani, 2009; Segurado and Tercero, 2009; Zegerman and Diffley, 2009, 2010; Labib and De Piccoli, 2011). Here we will review the current understanding of how the replication checkpoint senses and responds to replication stress, based mainly on the work carried out in the budding yeast model system. We will discuss recent evidence that sheds light on the checkpoint's essential function in promoting replication fork stability and on how cells inactivate checkpoint signaling to restore normal cell physiology. We will also consider the checkpoint from an evolutionary perspective and illustrate how it might act to suppress unrestrained proliferation and tumor progression in multicellular organisms.

REPLICATION FORK STALLING AND CHECKPOINT SIGNALING

Eukaryotic cells establish multiple replication forks in a time-regulated fashion due to the orderly activation of replication origins throughout S phase (Raghuraman et al., 2001). The Mcm2–7 complex replicative helicase unwinds the parental DNA helix, thus generating a ssDNA template for the replicative polymerases (Waga and Stillman, 1998). RPA stabilizes ssDNA tracks facilitating DNA synthesis and suppressing their engagement by recombination factors (Iftode et al., 1999). DNA polymerase ε is

thought to carry out leading strand synthesis, while DNA polymerase α and DNA polymerase δ primarily synthesize the lagging strand (Pursell et al., 2007). The replication fork is a complex structure in which DNA synthesis is coordinated with other DNA metabolic processes. A number of additional factors associate with replication forks to assist DNA polymerases processivity, lagging strand maturation, topological stress simplification, replisome stabilization, and coordination between replication and sister chromatid cohesion establishment (Tourriere et al., 2005; Gambus et al., 2006; Lengronne et al., 2006; Bermejo et al., 2007; Moldovan et al., 2007).

Faithful DNA replication requires that replication forks are processive and stable so that DNA synthesis is carried out with high fidelity throughout the genome. Replication fork progression can stall due to different causes. Template unwinding by replicative helicases can be counteracted by topological constraints, higher order DNA structures, or tightly DNA bound proteins (Azvolinsky et al., 2006; Bermejo et al., 2007; Labib and Hodgson, 2007). Additionally, damaged DNA and DNA synthesis inhibition owing to endogenous or exogenous factors may hamper replication fork progression. Agents generating DNA-topoisomerase adducts, intra-strand crosslinks or bulky DNA adducts can block the action of replicative helicases, whilst the progression of DNA polymerases can be impaired by the presence of base-adducts (as the ones generated by methylmethanesulphonate—MMS) or by direct inhibition of DNA synthesis (for instance through the depletion of dNTP pools induced by hydroxyurea) (Branzei and Foiani, 2010; Zegerman and Diffley, 2010; Ray Chaudhuri et al., 2012). Replication forks can also interfere with other DNA metabolism machineries. DNA and RNA polymerases compete for the same template during S phase and indeed replication machinery interference with the transcriptional apparatus has emerged as a major cause of fork collapse (for a recent review see Bermejo et al., 2012b). The mechanisms determining replication interference with transcription are not fully understood, though they might implicate clashes between replicative helicases and transcriptional machineries, topological interference with higher order chromatin structures established by co-transcriptional processes [such as gene loops or association with nuclear pore complexes (NPCs)] or engagement of aberrant RNA:DNA hybrids formed by the annealing of nascent RNAs (Deshpande and Newlon, 1996; Azvolinsky et al., 2009; Bermejo et al., 2009; Gomez-Gonzalez et al., 2011; Alzu et al., 2012).

When replication forks stall, a signaling cascade mediated by DNA damage checkpoint kinases is activated, spreading checkpoint signaling to a number of effectors that regulate diverse aspects of cell physiology. Factors involved in sensing and transducing the checkpoint signals generated at replication forks are highly conserved amongst eukaryotes (**Table 1**). Unless otherwise stated, we will refer in this review to the budding yeast homologs of these factors. At the center of the checkpoint signaling cascade are the phosphoinositide 3-kinases (PI3)-related Mec1 (HsATR) and Tel1 (HsATM) kinases (Weinert et al., 1994; Greenwell et al., 1995; Morrow et al., 1995; Savitsky et al., 1995; Bentley et al., 1996; Mallory and Petes, 2000; Paciotti et al., 2001). Human ATR and ATM are important to suppress malignant transformation

Table 1 | Replication checkpoint sensors and transducers.

Function	<i>S. cerevisiae</i>	<i>S. pombe</i>	<i>H. sapiens</i>
Sensors	Rfa1	Ssb1	RPA70
	Rfa2	Ssb2	RPA32
	Rfa3	Ssb3	RPA14
Apical kinases and interacting proteins	Mec1	Rad3	ATR
	Tel1	Tel1	ATM
	Ddc2	Rad26	ATRIP
Transducers	Mrc1	Mrc1	CLASPIN
	Rad9	Crb2	–
Effector kinases	Rad53	Cds1	CHK2
	Chk1	Chk1	CHK1
	Dun1	–	–

and can be found mutated in cancer cells (Kastan and Bartek, 2004). The highly conserved effector kinases Rad53 and Chk1 are directly targeted by PI3-related kinases and are responsible for the amplification of the checkpoint signal, as well as for the phosphorylation of key proteins that modulate different aspects of cellular physiology (Longhese et al., 2003).

Checkpoint activation in response to replication stress requires the presence of replication forks (Lupardus et al., 2002; Stokes et al., 2002). Indeed, it is thought that the generation of extended ssDNA tracks at replication forks is the main signal triggering replication checkpoint activation (You et al., 2002; Zou and Elledge, 2003). ssDNA at forks can be generated in response to replication inhibitors (such as hydroxyurea or aphidicolin), due to the uncoupling between DNA unwinding by helicases and the progression of DNA polymerases (Sogo et al., 2002; Byun et al., 2005), or by the uncoupling between leading and lagging strand polymerases due to the presence of damaged templates (Branzei and Foiani, 2009). Extended ssDNA tracks are readily coated by the single strand DNA-binding protein RPA complex (composed of Rfa1, Rfa2, and Rfa3) (Zou and Elledge, 2003) (**Figure 1A**), which recruits the apical kinase Mec1 to stalled forks through the action of its associated factor Ddc2 (Zou and Elledge, 2003). Upon recruitment to fork DNA, Mec1 phosphorylates several factors including Mrc1 (Alcasabas et al., 2001; Tanaka and Russell, 2001). Mrc1 is a structural component of the replication fork required for both DNA replication and checkpoint signaling (Osborn and Elledge, 2003; Szyjka et al., 2005; Tourriere et al., 2005). In response to replication stress Mrc1 acts as a signal transducer mediating full Rad53 kinase activation (Alcasabas et al., 2001). By analogy with the paradigmatic checkpoint transducer Rad9, Mrc1 is thought to act as a scaffold promoting Rad53 trans-autophosphorylation events (Pellicoli and Foiani, 2005; Chen and Zhou, 2009; Berens and Toczyski, 2012). Mec1 phosphorylates Rad53 in a Mrc1-dependent manner, and full kinase activity is achieved when different Rad53 molecules hyperphosphorylate each other before being released to reach their targets (Pellicoli and Foiani, 2005).

Mrc1 is necessary to sustain normal fork progression rates in the absence of genotoxic stimuli (Tourriere et al., 2005).

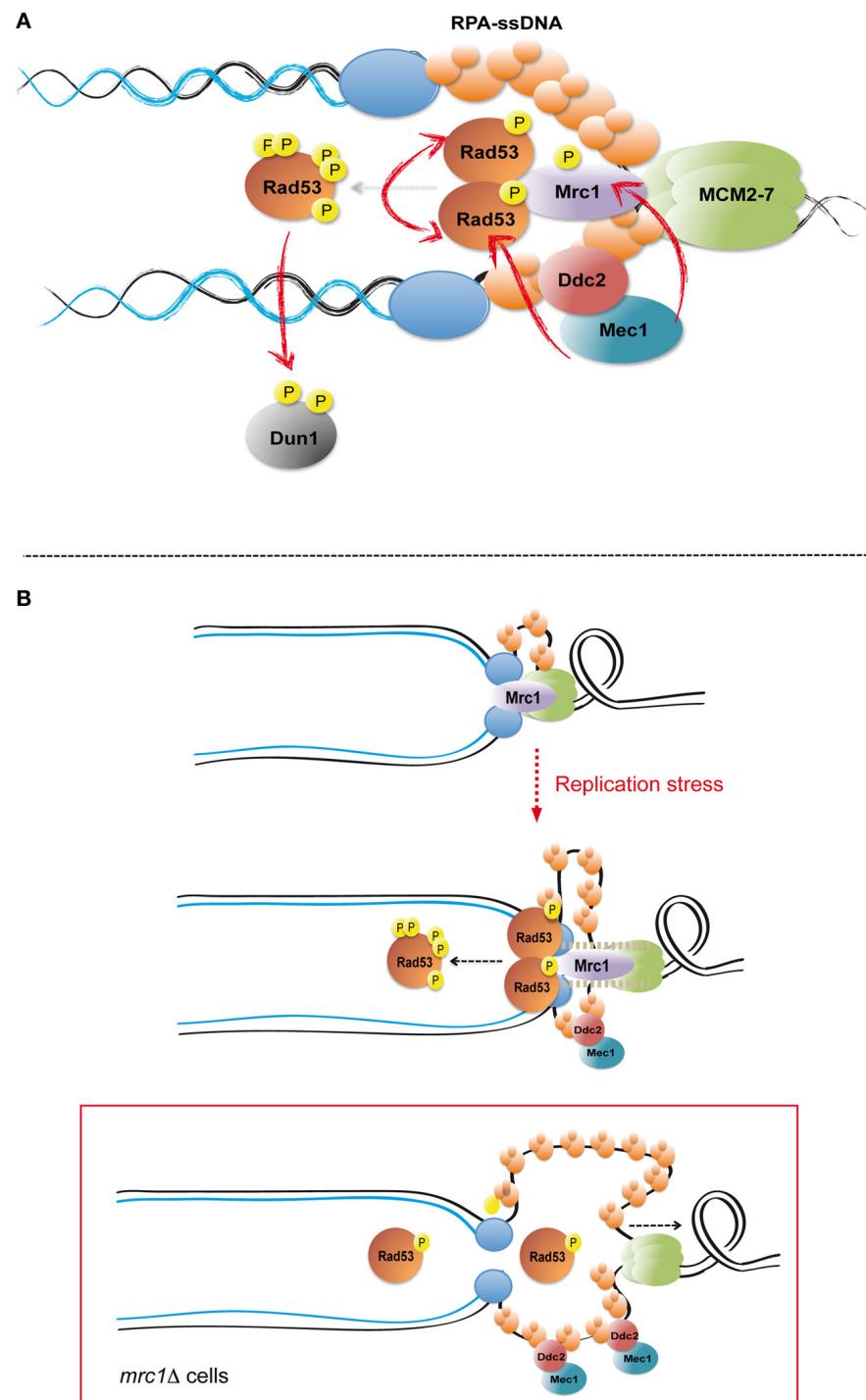


FIGURE 1 | Checkpoint activation in response to replication stress.

(A) Upon replication fork stalling ssDNA is generated by the replicative helicase—DNA polymerases uncoupling. RPA-ssDNA mediates the recruitment of the apical checkpoint kinase Mec1 to replication forks by the action of its associated factor Ddc2. Mec1 phosphorylates fork components, including the Mrc1 transducer, and the Rad53 effector kinase. Mrc1 serves as a scaffold promoting Rad53 trans-autophosphorylation events and full kinase activation. Rad53 phosphorylates and activates the Dun1 effector kinase. Red arrows indicate key phosphorylation events mediating checkpoint activation.

(B) Mrc1 is a replisome component that travels with replication forks in

unperturbed conditions. Following replication stress, Mrc1 prevents excessive DNA unwinding by restraining Mcm2–7 helicase progression, likely by physically tethering DNA helicases and polymerases. The tension generated between stalled polymerases and advancing helicases may determine conformational changes in Mrc1, thus promoting its function as a molecular scaffold necessary for Rad53 trans-autophosphorylation reactions. In Mrc1 ablated cells (*mrc1 Δ*), failure to restrain Mcm2–7 helicase leads to extensive DNA unwinding and ssDNA accumulation at replication forks, which does not directly result in Rad53 hyper-phosphorylation and full kinase activation due to the absence of Mrc1-mediated scaffolding.

In addition, Mrc1 prevents extensive uncoupling between helicase unwinding and DNA synthesis at stalled forks by somehow tethering helicases to DNA polymerases (Katou et al., 2003; Nedelcheva-Veleva et al., 2006). Importantly, Mrc1 interacts with polymerase ε catalytic subunit Pol2 in a checkpoint-dependent manner (Lou et al., 2008). Hence Mrc1 might act as a “molecular spring” sensing the physical connection between helicases and polymerases and at the same time preventing their uncoupling (**Figure 1B**). In this view, Mrc1 might suppress futile checkpoint signal amplification in forks to which Mec1 is recruited to ssDNA but the uncoupling between helicases and polymerases cannot be “physically” sensed.

Modulation of cellular physiology in response to replication stress is ultimately achieved through the regulation of a variety of effectors, which is mediated by phosphorylation events carried out by Mec1, Rad53, and Dun1 kinases (**Table 2**). Mec1 is thought to act locally by phosphorylating replication fork-associated (Smolka et al., 2007; Randell et al., 2010) and chromatin factors (Randell et al., 2010; Rodriguez and Tsukiyama, 2013). Several Rad53 targets are instead not localized at forks (Smolka et al., 2007; Chen et al., 2010), consistent with the notion that Rad53 may diffuse and propagate checkpoint signaling to distant effectors throughout the nucleus. Recently, the importance of checkpoint-mediated regulation of NPC and nuclear membrane-related processes in genome integrity maintenance has been revealed (Bermejo et al., 2011, 2012a) and it has been proposed that Rad53 might also regulate processes taking place in the cytoplasm (Enserink et al., 2006). Rad53 targets include the Dun1 kinase (Bashkirov et al., 2003), partially related to Rad53 (Zhou and Elledge, 1993; Rhind and Russell, 1998), which promotes the transcriptional induction of damage inducible genes and dNTP pool upregulation. The checkpoint response was originally considered a canonical signal transduction cascade composed by upstream sensors and a number of signal transducer kinases that regulate a large number of downstream effectors (Longhese et al., 2006). The picture though seems far more complex as factors can exert different roles in the cascade. For instance, Mec1 acts as a sensor and a signal transducer, but also directly phosphorylates effector proteins. Additionally, several sensors and transducers (as RPA complex proteins, Ddc2, or Mrc1) are directly phosphorylated by checkpoint kinases. Below we describe the better-characterized checkpoint-regulated processes contributing to maintain replication fidelity and genome integrity.

CHECKPOINT CONTROL OF S PHASE TRANSCRIPTION AND dNTP POOLS

The checkpoint response modulates cellular physiology to promote cell survival and preserve genome integrity. One of the earliest described checkpoint functions in response to replication stress is the delay of the progression through mitosis, which is achieved through direct modification of key cell cycle regulators and prevents the premature segregation of incompletely replicated chromosomes (Krishnan et al., 2004; Putnam et al., 2009; Palou et al., 2010). However, the majority of checkpoint-regulated events relevant for cell viability and genome integrity maintenance are thought to take place in S phase, including

Table 2 | Checkpoint kinases phosphorylation targets overview.

Checkpoint kinase	Regulated process	Targets
Mec1	DNA replication	Cdc2, Dpb4, Mcm4, Pol31, Psf1, Rfa1, Rfa2
	Checkpoint response	Ddc2, Dun1, Mec1, Mec3, Mrc1, Rad9, Rad17, Rad53
	DNA repair	Mlh1, Msh6, Rad23, Rad26, Rad55, Rtt107, Sae2, Slx4
	dNTP pools regulation	Ssn6
	Chromatin structure	Abf1, Hta1, Ies4, Isw2, Sin3, Sir4, Swi3
	NPC function	Hpr1, Nup2, Nup60
Rad53	Other	Cbf1, Cdc13, Nma111, Rif1, Spt7, Sum1
	DNA replication	Sld3, Rad27, Dbf4, Ctf4, Pol1
	Checkpoint response	Ddc1, Ddc2, Dun1, Mrc1, Rad9, Rad53, Tof1
	DNA repair	Exo1, Rad54, Rad55, Rtt107
	dNTP pools regulation	Crt1, Nrm1, Rnr3, Swi6
	Chromatin structure	Hhf1, Hho1, Hpc2, Esc1, Fun30, Itc1, Rph1, Snf2
Dun1	NPC function	Mlp1, Nsp1, Nup1, Nup2, Nup60, Hpr1
	Other	Mcd1, Plm2, Ycg1
	Checkpoint response	Dun1
	DNA repair	Nej1
	dNTP pool regulation	Crt1, Dif1, Rnr3, Sm1
	Chromatin structure	Hpc2, Rco1
Mrc1	NPC function	Mlp1, Nup159
	Other	Ecm21, Npl3, Sec3

upregulation of dNTP pools, inhibition of origin firing, stabilization of replication forks, and modulation of DNA repair. The functional meaning of other checkpoint-mediated effects such as modulation of transfer-RNA (tRNA) genes metabolism (Ghavidel et al., 2007; Nguyen et al., 2010) or the cellular redox state (Carter et al., 2005) are less clear, though repression of tRNA genes might counteract fork collapse by preventing forks clashing with the transcriptional apparatus (Nguyen et al., 2010).

Checkpoint kinases modulate the transcriptional program of cells experiencing replication stress (Smolka et al., 2012). The Dun1 kinase upregulates the transcription of damage inducible

genes by phosphorylating Crt1 (Huang et al., 1998) (**Figure 2A**). Crt1 binds to gene promoters and attracts the general transcriptional repressors Ssn6 and Tup1 (Huang et al., 1998). Dun1-dependent phosphorylation displaces Crt1 from promoter chromatin leading to the transcriptional activation of several genes, including *DUN1* itself and genes involved in dNTP synthesis, such as the ribonucleotide reductase (RNR) subunits encoding genes *RNR3*, *RNR2*, and *RNR4* (Zhou and Elledge, 1993; Zaim et al., 2005). Upregulation of *DUN1* expression feeds checkpoint signaling, thus contributing to strengthening Dun1-mediated control of dNTP levels (see below).

The MBF (Mlu1-box Binding Factor) heterodimeric transcription factor drives the expression of a variety of genes required for G1/S transition (Koch et al., 1993). MBF transcription is repressed upon S phase entry through the binding of the MBF-associated Nrm1 co-repressor (de Bruin et al., 2006). Nrm1 is a phosphorylation target of both Rad53 and its fission yeast ortholog Cds1 (de Bruin et al., 2008; Travesa et al., 2012) (**Figure 2A**). Rad53-mediated Nrm1 phosphorylation prevents it from binding to the MBF promoters, thus leading to

transcriptional upregulation of G1/S transition genes (Travesa et al., 2012). Genes whose expression is upregulated by Rad53 and Nrm1 in response to replication stress encode factors directly involved in DNA synthesis (i.e., *RNR1*, *RFA2*, *POL1*, *POL12*, *POL30*, *POL32*, *PRI2*, and *DPB2*), lagging strand maturation (i.e., *CDC9* and *RAD27*), replisome components, and accessory factors (i.e., *MRC1*, *CDC45*, *CTF4*, *CTF18*, *ECO1*, and *ELG1*) (Travesa et al., 2012).

The functional meaning of checkpoint-induced transcription is unclear. Preventing protein synthesis by cycloheximide treatment has little impact on cell survival following replication stress (Tercero et al., 2003). This observation led to the suggestion that checkpoint-induced transcription has a relatively small contribution to the stabilization of stalled replication forks. However, Nrm1 ablation confers resistance to hydroxyurea treatment (de Bruin et al., 2006, 2008) and Crt1 deletion mutants show increased viability following HU or MMS exposure (Shen et al., 2007 and our unpublished observations). Hence the transcriptional upregulation of key factors might be important to promote cell viability, perhaps by contributing to the

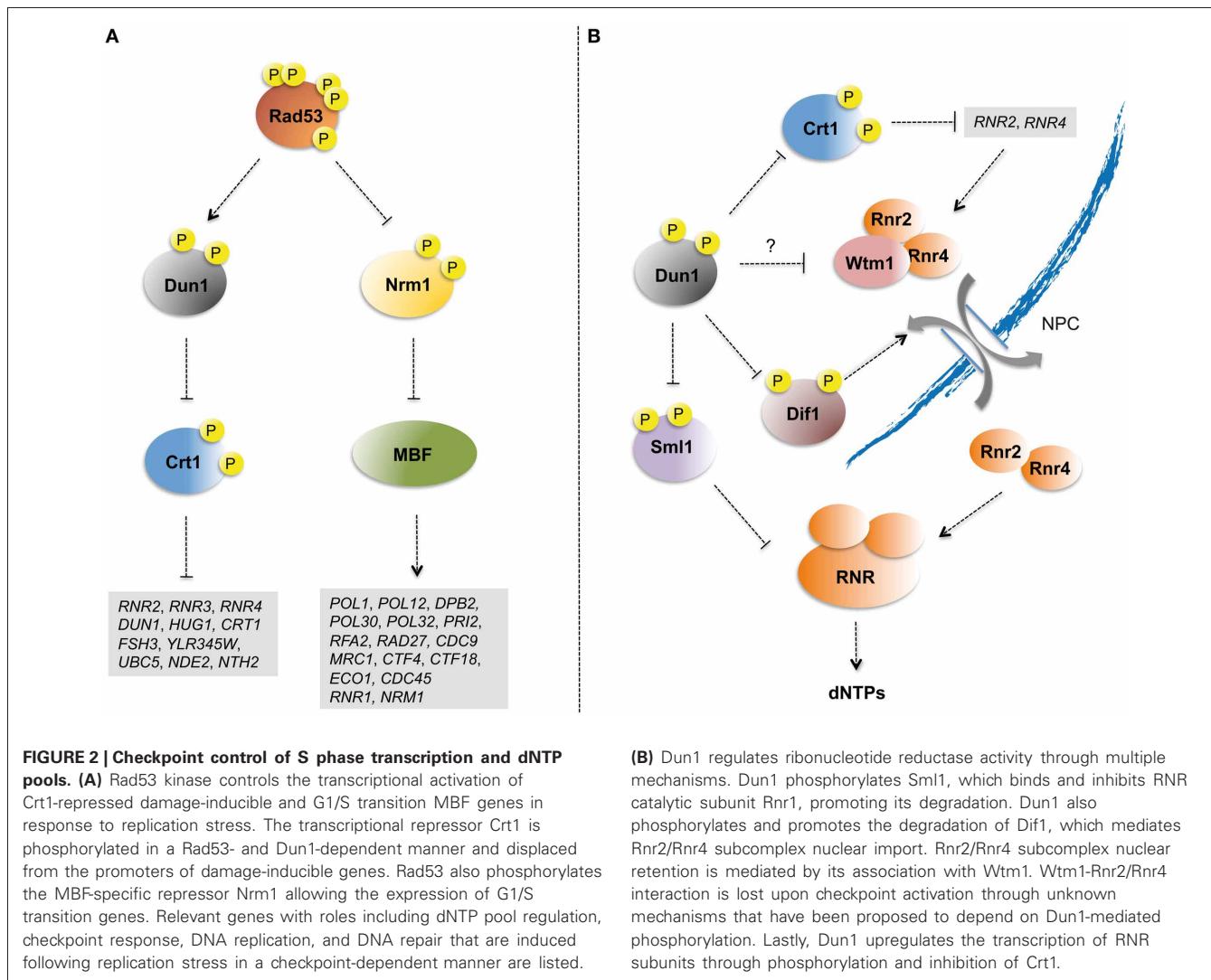


FIGURE 2 | Checkpoint control of S phase transcription and dNTP pools. (A) Rad53 kinase controls the transcriptional activation of Crt1-repressed damage-inducible and G1/S transition MBF genes in response to replication stress. The transcriptional repressor Crt1 is phosphorylated in a Rad53- and Dun1-dependent manner and displaced from the promoters of damage-inducible genes. Rad53 also phosphorylates the MBF-specific repressor Nrm1 allowing the expression of G1/S transition genes. Relevant genes with roles including dNTP pool regulation, checkpoint response, DNA replication, and DNA repair that are induced following replication stress in a checkpoint-dependent manner are listed.

stabilization of replication forks or the upregulation of dNTP levels. Further investigation will be required to elucidate this intriguing connection.

Tight regulation of dNTP pools is essential for cells to guarantee viability and prevent elevated mutagenesis rates (Chabes et al., 2003). Deregulation of the dNTP pool leads to genomic instability in yeast (Zhao et al., 2001) and mammalian cells (Bester et al., 2011). The replication checkpoint upregulates dNTP levels in response to replication stress, mainly through modulation of RNR activity (**Figure 2B**). RNR is a multimeric enzyme that catalyzes the reduction of ribonucleotides to deoxyribonucleotides, the rate-limiting step in dNTP synthesis. During most of the cell cycle the large catalytic subunit Rnr1 localizes to the cytoplasm, while Rnr2-Rnr4 subcomplex is nuclear (Yao et al., 2003). Dif1 directly binds and mediates the nuclear import of the Rnr2-Rnr4 subcomplex (Lee et al., 2008), which is retained in the nucleus through the action of Wtm1 (Lee and Elledge, 2006). Activation of checkpoint kinases leads to the re-localization of the RNR small subunits Rnr2-Rnr4 from the nucleus to the cytoplasm (Lee and Elledge, 2006). Following replication stress Dun1 phosphorylates Dif1 inducing its degradation (Lee et al., 2008) and Wtm1 interaction with the Rnr2-Rnr4 complex is abrogated (Lee and Elledge, 2006). Redistribution of Rnr2-Rnr4 to the cytoplasm favors its association with Rnr1 to constitute an active RNR complex and upregulate dNTP levels. As mentioned above, RNR subunits are transcriptionally induced in response to replication stress. A more striking effect is observed for *RNR3*. Rnr3 is an alternative catalytic subunit that can substitute Rnr1 to form active RNR complexes (Domkin et al., 2002) targeted by Rad53 (Smolka et al., 2007). *RNR3* has very low expression levels in the absence of genotoxic stresses and its protein levels following replication stress are relatively low as compared to those of Rnr1 (Li and Reese, 2001; Domkin et al., 2002). Hence, the functional role of Rnr3 in dNTP pool regulation remains unclear.

Checkpoint kinases also upregulate dNTP levels through Sml1, a small protein that directly binds to Rnr1 and inhibits RNR enzymatic activity (Zhao et al., 1998; Chabes et al., 1999). Phosphorylation of Sml1 by Dun1 triggers Sml1 degradation via a complex formed by the E2 ubiquitin-conjugating enzyme Rad6, the E3 ubiquitin ligase Ubr2, and the accessory factor Mub1 (Zhao and Rothstein, 2002; Andeson et al., 2010). Upregulation of dNTP pools could contribute to stabilizing replication forks by directly increasing polymerase processivity or by facilitating a more efficient repair of lesions blocking fork progression. Importantly, Mec1 and Rad53 are thought to regulate dNTP pools in unperturbed S phase, as the lethality of *MEC1* or *RAD53* deletion is suppressed by Sml1 ablation (Zhao et al., 1998), and defective dNTP pool regulation in checkpoint mutants results in spontaneous fragility of hard-to-replicate genomic regions (Cha and Kleckner, 2002).

CHECKPOINT CONTROL OF REPLICON DYNAMICS AND FORK STABILITY

Cells experiencing replication stress modulate chromosomal replication through at least two checkpoint-dependent mechanisms: the stabilization of stalled replication forks and the block of origin firing. Replication origins fire with a somewhat pre-defined

timing throughout unperturbed S phases (Raghuraman et al., 2001). In response to replication stress origin firing is regulated by checkpoint kinases that mediate the repression of late and dormant origins (Santocanale and Diffley, 1998; Shirahige et al., 1998). This effect is directly mediated by Rad53, which phosphorylates Dbf4 and Sld3 proteins thus short-circuiting the two alternative Dbf4-dependent kinase (DDK) and cyclin-dependent kinase (CDK) pathways that promote origin firing in S phase (Lopez-Mosqueda et al., 2010; Zegerman and Diffley, 2010). Interestingly, dormant origin derepression also takes place when a double strand break (DSB) is induced at a neighboring HO-endonuclease sequence in the budding yeast mating type locus (Doksani et al., 2009). HO-break mediated origin derepression occurs even when Rad53 is fully activated owing to HU treatment. Hence alternative mechanisms, perhaps involving chromatin structure changes, might bypass checkpoint control on origin firing. Prevention of late origin firing in response to replication stress seems to have obvious advantages for the cell. When forks stall due to reduced dNTP levels, establishing more replication forks at late origins would further increase dNTPs demand. In the presence of damaged templates, limiting late origin firing would prevent additional forks to stall by running into DNA lesions. However, the inability to prevent late origin firing is not thought to be the major cause of cell lethality in checkpoint mutants experiencing replication stress as *mec1-100* mutants, which fail to prevent late origin firing, are not sensitive to HU or MMS treatments (Tercero et al., 2003).

The most crucial function exerted by checkpoint kinases is the protection of fork stability (Lopes et al., 2001; Tercero and Diffley, 2001; Sogo et al., 2002), which has been argued to account for the maintenance of cell viability following replication stress (Segurado and Diffley, 2008). In checkpoint mutants, replication forks fail to resume DNA synthesis after removal of replication stress-inducing drugs (Desany et al., 1998) and accumulate DNA breaks (Feng et al., 2006; Raveendranathan et al., 2006; Feng et al., 2011). The loss of replication fork functional integrity accompanied by structural alterations of replication intermediates is usually termed fork collapse and it is thought to be a major cause of gross chromosomal rearrangements in checkpoint-deficient cells (Myung et al., 2001; Myung and Kolodner, 2002; Admire et al., 2006). Fork stability defects also result in an increased incidence of malignant tumors (Kawabata et al., 2011). Currently the checkpoint-mediated mechanisms counteracting fork collapse are not fully understood, though fork-protecting pathways may interplay.

Collapsed replication forks in checkpoint deficient cells are characterized by the accumulation of abnormal replication intermediates (Lopes et al., 2001; Cotta-Ramusino et al., 2005). Prominently, checkpoint mutants exhibit forks in which nascent strands re-anneal to generate four-way junctions, often referred to as reversed forks (Sogo et al., 2002). Formation of reversed forks is promoted by the accumulation of torsional stress both *in vitro* and *in vivo* (Postow et al., 2001a,b; Bermejo et al., 2011; Ray Chaudhuri et al., 2012). Positive supercoiling generated by DNA unwinding at the replication fork tends to re-anneal parental DNA strands (Wang, 2002), thus regressing the fork branching point, and strip-off the nascent strands. Nascent strands can

in turn pair due to their sequence homology (**Figure 3A**). Fork reversal driven by positive supercoiling is favored *in vitro* by protease treatments that eliminate replisome components from fork DNA, suggesting that the association of replisome factors with nascent DNA strands might counteract the topological transitions leading to fork reversal (Postow et al., 2001b). Recent evidence suggests that the replication checkpoint modulates chromosome architecture and can attenuate the impact of positive supercoiling on stalled forks (Bermejo et al., 2011; Dion et al., 2012; Mine-Hattab and Rothstein, 2012). Rad53 phosphorylates the

Mlp1 nucleoporin, which mediates the association of RNA polymerase II transcribed genes to NPCs in a phenomenon known as gene gating (Kohler and Hurt, 2007). Transcribed chromatin associating with the fixed NPC structure would prevent the rotation of DNA strands around each other establishing a barrier to the diffusion of topological changes (Koster et al., 2010). In this view, positive supercoiling would tend to progressively accumulate as replication forks approach transcribed genes (**Figure 3B**). Upon treatment with HU, transcribed genes association with NPCs is released in a checkpoint-dependent manner (Bermejo

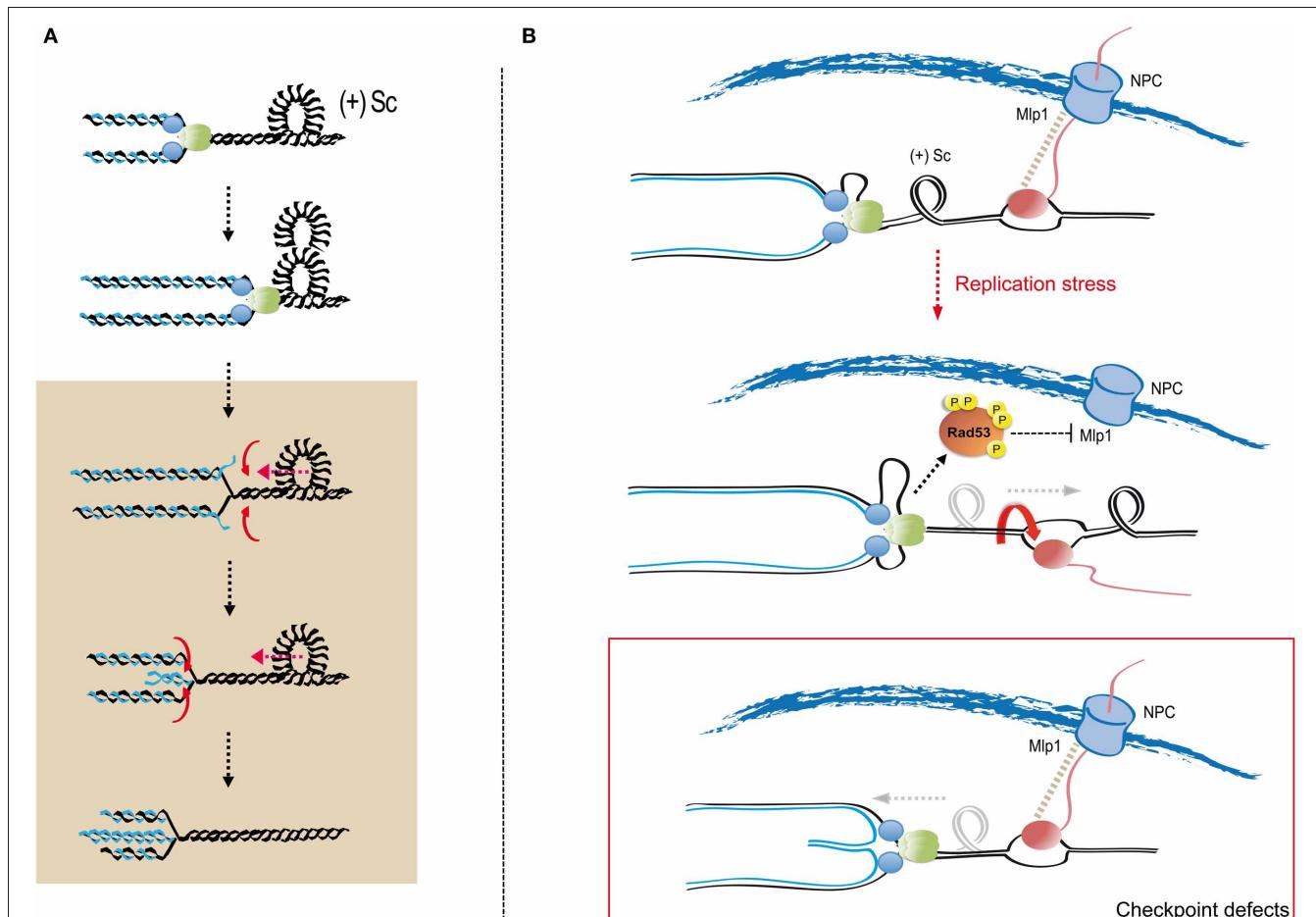


FIGURE 3 | Replication fork reversal and checkpoint-mediated topological simplification at transcribed regions. **(A)** DNA double helix unwinding during replication generates torsional stress that can accommodate as positive supercoiling (+Sc) ahead of replication forks. Progressively accumulating positive supercoiling provides the driving force for replication fork reversal; particularly upon the dissociation of replisome components from fork DNA. Positive supercoiling can be re-accommodated by re-winding of the parental strands, which results in the regression of the fork branching point and the extrusion of newly synthesized strands (in blue). Newly synthesized DNA strands annealing, driven by sequence homology, leads to the formation of four-way cruciform junctions known as reversed forks or *chicken feet*. Reversed forks can branch-migrate due to further positive supercoiling-driven parental strand re-annealing. Replisome components are represented as green and blue circles. The gray box delimits aberrant transitions leading to fork reversal. **(B)** Activation of

checkpoint kinases counteracts gene gating. In S phase, replication forks engage RNA polymerase II-transcribed genes, which associate to the inner basket of NPCs through the action of co-transcriptional protein complexes and key nucleoporins (including Mlp1) in a process known as “gene gating.” Gated genes behave as barriers to topological stress diffusion as they counteract the rotation of helix strands around each other, thus favoring the accumulation of positive supercoiling ahead of approaching replication forks. Following replication stress Rad53 phosphorylates Mlp1 thus releasing transcribed genes from their association to the nuclear pores. Disengagement of transcribed genes permits DNA rotation and the diffusion of topological stress away from replication forks. In checkpoint deficient cells topological barriers persist, favoring positive supercoiling relaxation through reversal of stalled forks. Replisome components and the transcriptional apparatus are represented as green/blue and pink circles, respectively.

et al., 2011), likely removing topological barriers that drive local positive supercoiling accumulation and promote fork reversal (**Figure 3B**). Reversed forks cannot sustain DNA synthesis and might represent a terminal step of fork stalling accounting for the loss of viability of checkpoint mutants upon treatment with replication stress inducing drugs. Genetic contexts suppressing fork reversal positively affect checkpoint deficient cells survival upon HU treatment (Bermejo et al., 2011) and mechanisms re-starting reversed forks in the absence of checkpoint kinases have not been described. It is, however, unclear whether fork reversal is necessarily a terminal event in eukaryotic cells (Ray Chaudhuri et al., 2012).

Nucleolytic processing activities also engage collapsed replication forks. Checkpoint mutants experiencing replication stress induced by dNTP pool depletion accumulate forks with extended ssDNA gaps and replication bubbles in which one of the nascent strands is absent (Sogo et al., 2002). The formation of gapped and hemireplicated molecules is partly dependent on the action of the Exo1 nuclease (Cotta-Ramusino et al., 2005). Ablation of Exo1 also reduces the accumulation of reversed forks (Cotta-Ramusino et al., 2005), suggesting that Exo1 might either promote their resolution by resecting reversed strands or the formation of extended ssDNA gaps precluding nascent strand re-annealing. Exo1 induces fork instability and lethality in checkpoint deficient cells that replicate damaged templates (Segurado and Diffley, 2008). Exo1 is phosphorylated by Rad53 (Smolka et al., 2007) and it has been suggested that targeting by Rad53 might down-regulate Exo1 activity (Morin et al., 2008). This is in agreement with the notion that the checkpoint suppresses Exo1-mediated processing of normal and/or aberrant DNA structures at stalled replication forks, thus preventing fork breakdown (Segurado and Diffley, 2008). Exo1-dependent processing of collapsed forks is likely to prime unscheduled recombination events giving rise to gross chromosomal re-arrangements in checkpoint deficient cells (Myung and Kolodner, 2002; Kaochar et al., 2010). It has been proposed that further nucleolytic cleavage could target collapsed forks contributing to the formation of DNA breaks or as part of DNA repair attempts (Branzei and Foiani, 2009). The identity of the factors mediating such processing and the implication of the checkpoint in suppressing their action remain to be discovered.

Several replisome components including DNA polymerase α and δ subunits, as well as components of the Mcm2–7 and GINS helicase complexes are direct targets of Mec1 and Rad53 phosphorylation (Smolka et al., 2007; Chen et al., 2010; Randell et al., 2010) (**Table 2**) and the association of replicative polymerases and the Mcm2–7 helicase complex to stalled replication forks is impaired in checkpoint kinases mutants (Cobb et al., 2003, 2005; Lucca et al., 2004). These observations led to the suggestion that checkpoint kinases regulate the tethering of essential replisome components to fork DNA and that the loss of this tethering is the reason for checkpoint mutants inability to resume DNA synthesis. In agreement with this hypothesis, some replisome factors, such as the Mcm2–7 complex, cannot be re-loaded to replication forks (Labib et al., 2000), nor do efficient mechanisms for re-loading essential replication factors to collapsed forks seem to operate (Zegerman and Diffley, 2009). A recent study showed

that the association between replisome components isolated by immunoprecipitation following genotoxic treatment is equivalent in wild type cells and checkpoint mutants (De Piccoli et al., 2012), suggesting that replisomes do not suffer gross structural alterations as a result of fork collapse. The same study showed that DNA polymerase α and Mcm2–7 complex components remained associated to a large fraction of replication forks following HU treatment in the absence of checkpoint kinases, raising the possibility that lack of phosphorylation of replisome components, rather than dissociation from replication forks, accounts for the inability of cells to re-start DNA synthesis (De Piccoli et al., 2012). Interestingly, forks from which replisome components are lost correlate with those emanated from earliest origins, though the specific determinants of the susceptibility of these forks to replisome dissociation are unclear.

The relative contribution of replisome destabilization, replication fork reversal, and the nucleolytic processing of replication intermediates to the loss of replication fork functionality is unclear. It is likely that the three processes interplay to promote fork collapse if not effectively suppressed by checkpoint kinases (**Figure 4**). It is tantalizing to speculate that checkpoint kinases might somehow contribute to maintain the association of DNA polymerases with nascent DNA chains, perhaps by restraining helicase activity, or DNA polymerases processivity through direct phosphorylation events. DNA polymerases might be physically displaced from the 3' termini of nascent strands by an excessive tracking of the replisome along the parental DNA, thus losing their capacity to continue DNA synthesis. The mechanical stress imposed by positive supercoiling may also contribute to displacing DNA polymerases from 3' termini by peeling-off the nascent strands from the parental template. Nucleolytic processing at forks might in turn be favored by the exposure of the termini of nascent strands upon replisome dislodgement or fork reversal (**Figure 4**). Nucleolytic cleavage of ssDNA or branched structures could eventually generate discontinuities allowing the dissociation of replisome factors topologically linked to DNA (such as the Mcm2–7 complex or PCNA rings), thus accounting for the replisome loss observed in checkpoint mutants at early established replication forks (Cobb et al., 2003, 2005; Lucca et al., 2004; De Piccoli et al., 2012). Further work will be required to understand the checkpoint-mediated mechanisms protecting replication forks and their relative impact on genome integrity maintenance in response to different replication stress-inducing agents in detail.

CHECKPOINT SIGNALING REVERSION AND RESTORATION OF NORMAL CELLULAR PHYSIOLOGY

As discussed above, checkpoint activation in response to replication stress has a profound impact on several cellular processes, including modulation of the transcriptional program, replicon dynamics, and cell cycle progression. Checkpoint kinases phosphorylate and/or regulate the expression levels of a large number of factors. Once replication stress is overcome, normal cellular physiology needs to be restored. This requires shutting-off the checkpoint signaling cascade, as well as the reversion of posttranslational modifications and expression level changes of checkpoint transducers and effectors.

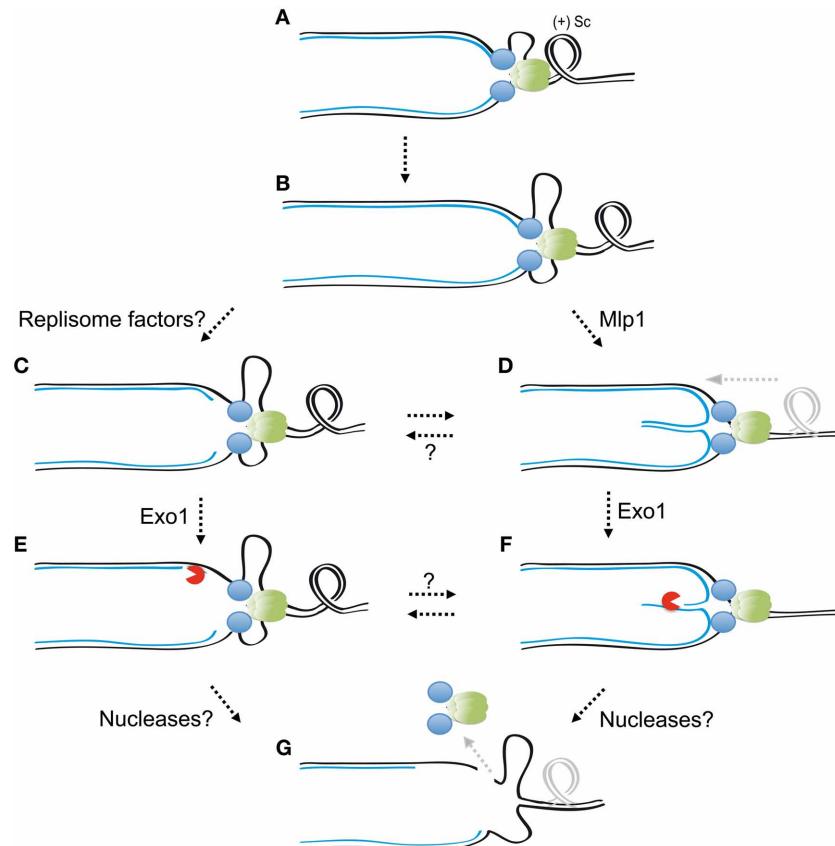


FIGURE 4 | Interplay between checkpoint-mediated mechanisms counteracting replication fork collapse. In unperturbed conditions replication fork advancement generates positive supercoiling (A). Fork stalling and helicase/polymerases uncoupling generate extended ssDNA tracks triggering checkpoint activation (B). Replisome factors targeting by checkpoint kinases might limit replisome tracking and thus prevent the dissociation of DNA polymerases from nascent strands termini (C). Checkpoint kinases also inhibit Mlp1 function, allowing positive supercoiling diffusion, and counteracting replication fork reversal (D). In the absence of

checkpoint kinases Exo1 could access exposed nascent strands termini and generate extended ssDNA tracks (E,F). Further nucleolytic processing of ssDNA tracks or branch cleavage activities could determine the formation of DNA breaks, which may in turn favor replisome dissociation from fork DNA (G). The combined action of these checkpoint-suppressed events likely contributes to the loss of functional integrity of stalled forks. Factors phosphorylated and potentially inhibited by checkpoint kinases to suppress abnormal fork transitions are indicated. Replisome components are represented as green circles.

A first mechanism contributing to checkpoint signaling inactivation is likely to be the elimination of upstream signals recognized by checkpoint sensors at replication forks. Upon resumption of DNA synthesis, ssDNA tracks shorten as DNA polymerases catch up with each other or with helicases, thus limiting further Ddc2-mediated recruitment and activation of Mec1. Interruption of Mec1 signaling may be sufficient to shut-off the checkpoint response, as Mec1 activity downregulation by overexpression of a dominant negative *MEC1* allele results in premature Rad53 dephosphorylation (Paciotti et al., 2001). However, an exclusively passive mechanism is unlikely to account for the rapid checkpoint inactivation observed after the removal of replication stress-inducing drugs (Travesa et al., 2008), indicating that mechanisms exist that actively revert checkpoint signaling.

A straightforward way to actively interrupt checkpoint signaling is to reverse the phosphorylation events mediated by checkpoint kinases. Work over the last years has focused on the inactivation of Rad53 kinase through dephosphorylation, which

is quickly achieved upon the removal of replication stress inducing agents. Rad53 dephosphorylation tightly correlates with the downregulation of its kinase activity and does not require protein synthesis (Pellicoli et al., 1999). Budding yeast phosphatases Ptc2, Ptc3, and Pph3/Psy2 are required for Rad53 dephosphorylation following replication stress (O'Neill et al., 2007; Szyjka et al., 2008). The Pph3 phosphatase and its regulatory subunit Psy2 form a complex that dephosphorylates Rad53 *in vitro* and it has been suggested that they directly inactivate Rad53 (O'Neill et al., 2007). It is unclear, however, whether Pph3 has other important targets in checkpoint inactivation (Keogh et al., 2006). Ptc2 and Ptc3 are type 2C protein phosphatases with redundant functions in the checkpoint response (Leroy et al., 2003). Ptc2 and Ptc3 can bind Rad53 and directly mediate its dephosphorylation (Leroy et al., 2003; Guillemain et al., 2007). Both *ptc2*, *ptc3*, and *psh3/psy2* deletion mutants accumulate hyperphosphorylated Rad53 upon treatment with HU or MMS (Travesa et al., 2008). Ptc2/Ptc3 human homologs Wip1/PPM1D also play important

roles in the reversal of DNA damage checkpoint responses by dephosphorylating and inactivating checkpoint components (for review see Heideker et al., 2007; Clemenson and Marsolier-Kergoat, 2009).

Interestingly, checkpoint inactivation has different genetic requirements upon continuous exposure to (adaptation) or during the recovery from replication stress. Pph3/Psy2 complex mediates Rad53 dephosphorylation both during adaptation and recovery from HU or MMS treatments, while Ptc2 and Ptc3 are dispensable during recovery (Travesa et al., 2008). The Glc7/protein phosphatase 1, recently shown to be involved in checkpoint inactivation, acts both during adaptation and recovery following HU treatment, but is dispensable during MMS exposure (Bazzi et al., 2010). These differential genetic requirements for checkpoint inactivation point to the existence of distinct modifications of checkpoint factors that might be crucial for checkpoint function in different cellular contexts or upon different replication stress-inducing stimuli. Recent observations indicate that checkpoint inactivation can also be achieved through degradation or cellular sorting of checkpoint transducers and/or effectors. Mammalian CHK1 effector kinase is inactivated through proteasome-dependent downregulation upon ATR-mediated phosphorylation, which leads to both CHK1 activation and its marking for degradation (Zhang et al., 2005). Furthermore, the Mrc1 human homolog CLASPIN is targeted for degradation in response to HU treatment, thereby promoting CHK1 inactivation (Mailand et al., 2006; Mamely et al., 2006; Peschiaroli et al., 2006).

Reversion of checkpoint-induced changes in the transcriptional program is promoted by the establishment of negative feedback loops (Smolka et al., 2012). As mentioned above, Crt1 and Nrm1 are phosphorylated by checkpoint kinases, which remove them from damage inducible and MBF targets gene promoters (Huang et al., 1998; Travesa et al., 2012). *CRT1* and *NRM1* promoters are bound by Crt1 and Nrm1, respectively, and therefore their transcription is upregulated along with that of other damage inducible genes upon checkpoint activation. Overexpression of Crt1 and Nrm1 provides a simple mechanism to limit checkpoint-mediated transcriptional changes, as accumulating Crt1 and Nrm1 might escape regulation by checkpoint kinases to mediate the repression of the relevant genes. This mechanism might be particularly efficient upon concomitant inactivation of checkpoint signal transduction, as newly synthesized Crt1 and Nrm1 would not be inhibited by checkpoint kinases.

Untimely persistence of checkpoint signaling might impact on replication dynamics. It has been suggested that checkpoint kinases slow down replication fork progression rates by directly phosphorylating replisome components (Labib and De Piccoli, 2011). Consistently, DNA synthesis resumption at MMS stalled forks is severely impaired in cells lacking Pph3 phosphatase (Szyjka et al., 2008). Such slowly progressing forks would need to traverse longer genomic regions before fusing, as persistent checkpoint signaling may also suppress late origin firing. Furthermore, the fidelity of DNA synthesis at these slow-progressing forks might be additionally compromised by the persistence of abnormally elevated dNTP pools. Therefore, a failure to promptly

inactivate the checkpoint response once cells overcome replication stress might greatly impact genome integrity. In the future, it will be interesting to analyze which cellular mechanisms revert checkpoint signaling following different kinds of replication stress induced by diverse chemotherapeutic agents, as well as to study the impact of checkpoint inactivation defects on malignant transformation and cancer development.

CHECKPOINT EVOLUTION AS AN ANTICANCER BARRIER IN MULTICELLULAR ORGANISMS

Apical checkpoint kinases share homology with the PI3-related TOR kinases, which modulate cellular metabolism in response to nutrient availability (Lovejoy and Cortez, 2009). As mentioned above, budding yeast checkpoint kinases play an essential role in regulating dNTP pools even in the absence of replication stress (Zhao et al., 1998). It is therefore reasonable to think that the ancestral role of checkpoint kinases might have been to modulate the cellular metabolism in order to readily meet the elevated demand for dNTPs imposed by DNA replication during S phase. This function may have become crucial to ensure DNA replication fidelity in unicellular eukaryotes in which growth and proliferation greatly depend on nutrient availability (Alberghina et al., 2012). In this view, checkpoint kinases might have become progressively specialized in sensing and responding to stimuli requiring the upregulation of dNTP levels such as replication stalling by exogenous toxins or the repair of DNA damage. The evolutionary advantage of being able to survive such genotoxic insults might have favored checkpoint kinases gaining control over other cellular processes essential for replication integrity such as the control of cell cycle progression, replication origin firing, or replication fork stabilization. Checkpoint control of these functions is conserved in multicellular organisms (Jackson and Bartek, 2009; Ciccia and Elledge, 2010), although in higher eukaryotes the checkpoint response regulates mechanisms driving cells out of proliferating pools such as senescence or apoptosis. This might relate to the fact that cell proliferation decisions in higher eukaryotes are integrated at the organism level and are relatively independent from environmental nutrient availability.

In recent years it has emerged that the checkpoint response behaves as a barrier preventing tumorigenesis at early stages of cancer development (Bartek et al., 2007a; Halazonetis et al., 2008). This idea is supported by the observations that tumor cells, unlike other highly proliferating cells, show constitutively activated checkpoint kinases and markers of DNA breakage (Bartkova et al., 2005; Gorgoulis et al., 2005) and that oncogene activation induces replication stress, fork collapse, and formation of DNA breaks (Bartkova et al., 2006; Di Micco et al., 2006). Replication stress and DNA damage in this context may emerge from massive interference of replication forks with unscheduled oncogene-induced transcription (Bermejo et al., 2012b). In early pre-invasive lesions the checkpoint response is thought to promote pre-malignant cells removal from proliferating pools into senescence or apoptosis (Bartek et al., 2007b). Mutations or epigenetic silencing of checkpoint genes might result in an increased accumulation of DNA breaks owing to faulty fork stabilization, as well as to the loss of the checkpoint-mediated restraints to proliferation. Hence, cancer cells could proliferate and expand at

the expense of an increased genomic instability, thus accelerating tumorigenesis.

Future research should focus on integrating the current insight on checkpoint-mediated replication fork protection with a deeper knowledge on the determinants driving fork collapse in cells experiencing oncogene-induced replication stress. It will also be interesting to explore the connections between checkpoint inactivation mechanisms, replication dynamics, and genome integrity maintenance; and how checkpoint signaling modulation might interplay with the checkpoint function in suppressing cell proliferation to act as an anticancer barrier.

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LIM protein Ajuba participates in the repression of the ATR-mediated DNA damage response

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LIM proteins constitute a superfamily characterized by the presence of a LIM domain, known to be involved in protein–protein interactions. Our previous work has implicated members of the Zyxin family of LIM proteins, namely TRIP6 and LPP, in the repression of the DNA damage response (DDR) at telomeres. Here, we describe a role for Ajuba, a closely related LIM molecule, in repressing the ATR-mediated DDR. We found that depletion of Ajuba led to apparent delays in the cell cycle, accompanied with increased Rb phosphorylation, Chk1 phosphorylation, induction of p53, and cell death. Ajuba could be found in a complex with replication protein A (RPA), and its depletion led to RPA phosphorylation, known to be an early event in ATR activation. We propose that Ajuba protects against unscheduled ATR signaling by preventing inappropriate RPA phosphorylation.

Keywords: LIM protein, Ajuba, ATR, DNA damage, RPA

INTRODUCTION

Maintenance of genomic integrity is essential for accurate transmission of genetic information and cell viability. DNA damage by endogenous and exogenous agents can lead to genomic instability, itself a causative factor in early human tumorigenesis (Bartkova et al., 2005; Gorgoulis et al., 2005). Cells have specific checkpoints to detect damaged or abnormally structured DNA and allow for activation of repair mechanisms, or activation of apoptosis (reviewed in Ciccia and Elledge, 2010). Checkpoints operate at distinct points in the cell cycle to check for DNA lesions and act to delay transitions from both G1 to S phase and G2 to M phase, as well as within S (reviewed in Zhou and Bartek, 2004). In addition, checkpoints can monitor cells for M phase exit. In order to counteract and repair the DNA damage, the cell elicits a DNA damage response (DDR), under the control of signaling kinases part of the PIKK family, ATM, ATR, and DNA-PK (Ciccia and Elledge, 2010). These DDAs are organized pathways consisting of specific steps of damage sensing, transduction of a damage signal, and induction and recruitment of repair proteins to the damaged sites. Of those, ATM is important for the repair of double strand breaks and is not essential for viability, but controls an important tumor suppressor pathway. ATR however, is essential, and is activated upon types of damage generated by UV irradiation, such as Thymine dimers, or DNA replication defects in S phase such as replication fork collapses or accumulation of single stranded DNA. It has been proposed that the essential nature of the ATR pathway is caused by the necessity to repair spontaneous damage occurring during a normal S phase, which would lead to an intolerable level of damage if left unrepaired (see Hurley and Bunz, 2007 and references therein).

Abbreviations: ATM, ataxia-telangiectasia mutated; ATR, ataxia-telangiectasia and Rad3-related; Chk1, Checkpoint 1; Chk2, Checkpoint 2; DDR, DNA damage response; FACS, fluorescence activated cell sorting; LIM, Lin-1, Isl-1, Mec-3 domain; RPA, replication protein A.

The ATM and ATR kinases have a number of target substrates, among which the kinases Chk1 and Chk2, which are important for the respective cellular responses to the damage. Chk2 is an ATM target and is phosphorylated after ionizing radiation. Chk1, on the other hand, is a direct target of ATR, and is phosphorylated during replication stress or UV irradiation. ATR, therefore, is the kinase involved in responding to endogenous lesions or errors occurring through the action of replication forks during a normal S phase (Sorensen et al., 2004; Vassil et al., 2009).

During DNA replication, the single stranded DNA produced is bound by replication protein A (RPA), a complex of three subunits, RPA70, RPA32, and RPA14, which binds single DNA through OB fold structural motifs in a sequence-independent manner (Wold, 1997). RPA is a central molecule in the activation of ATR. The RPA32 subunit is phosphorylated after damage, and recruits ATRIP, itself required for the activation process (Zou and Elledge, 2003). Upon a sustained DDR, the ATM pathway is activated leading to activation of p53 and further checkpoint delays in the cell cycle.

Another event that can trigger DDR is telomere deprotection. Mammalian telomeres consist of TTAGGG tandem repeats that end with a 3' overhang (Palm and de Lange, 2008). A six-protein complex called shelterin binds to the telomeric repeats, and, as part of this complex, TRF2 was found to prevent inappropriate activation of ATM (Karlseder et al., 2004). Another shelterin protein, POT1, directly binds the telomeric single stranded overhang and protects against ATR activation (Lazzerini Denchi and de Lange, 2007; Palm et al., 2009).

Our laboratory has previously shown that members of a distinctive class of molecules called LIM proteins are implicated in telomere protection by repressing DDR at telomeres (Sheppard and Loayza, 2010; Sheppard et al., 2011). Specifically, LIM proteins TRIP6 and LPP belong to the Zyxin family (Kadrmas and Beckerle, 2004) and interact with the shelterin complex to prevent DDR activation at telomeres. The Zyxin family is characterized by

the presence of three LIM domains present at the C-terminus, with each domain containing of two Zinc fingers, and a unique pre-LIM region at the N-terminus. They also posses a nuclear export signal close to the N-terminus and hence can shuttle between the nucleus and the cytoplasm (Wang and Gilmore, 2001). The LIM protein TRIP6, in particular, was shown to interact with OB-fold-containing protein POT1 through the C-terminal LIM domains (Sheppard and Loayza, 2010).

Here, we are investigating the role of Ajuba, a closely related LIM protein, part of the Zyxin family. We found that Ajuba also participates in the repression of the DDR, but in a genome-wide fashion. We describe the role of Ajuba as a repressor of the ATR pathway, and show that this molecule is in a complex with RPA and prevents unscheduled phosphorylation of RPA32. We propose a model in which Ajuba controls the transition between local activation of ATR during a normal S phase and the global ATR activation occurring after extensive DNA damage.

MATERIALS AND METHODS

CELL LINES AND ANTIBODIES

The cell lines used were HTC75 cells and IMR90. The HTC75 cell line is a HT1080 derivative described in (van Steensel and de Lange, 1997). IMR90 cells were obtained from the ATCC at population doubling 21. The cells were grown in DMEM supplemented with 1% penicillin and streptomycin, 10% BCS for HTC75, and 10% FBS for IMR90 cells. The Ajuba antibody was obtained from Abcam (AB64451). The Ajuba serum was generated against a peptide conjugated to KLH and used for immunization into rabbits, as per the protocol set by the manufacturer (BioSynthesis, Lewisville, TX, USA). The peptide was: NH₂-CPRGATGGPGDEPLEPAREQGSLDA-OH for Ajuba. The antibodies for Rb-pS807/811(9308), PARP (9542), p53-p-Ser20 (9287), Cyclin A2 (4656), Chk1-p-S345 (2348), Chk1-p-S296 (2349) were obtained from Cell signaling. The total Chk1 antibody was purchased from Sigma-Aldrich (C9358). The p53 antibody was acquired from Millipore (04-1083). The RPA-p-T21 antibody was purchased from Abcam (AB109394). The GAPDH antibody was obtained from Santa Cruz (sc-32233). The p53BP1 (NB100-304) and RPA2 (9A1) antibody was purchased from Novus.

DEPLETION BY siRNA

HTC75 cells and IMR90 cells were maintained in DMEM (Invitrogen)/1% penicillin and streptomycin/10% FBS. Ajuba specific siRNAs were synthesized by Dharmacon RNA Technologies. For Ajuba RNAi, double-stranded siRNA were designed to target the following sequences: Ajuba si#1 siRNA 5'-CCAAAUGGAUUGUGGAAGAUU-3', Ajuba si#2 siRNA 5'-GGGAAAGAGGUCAGAUUAUU-3', and Ajuba si#3 siRNA 5'-GCAGCUGAGUGAUGAGGAUU-3'. The cells were transfected using Lipofectamine (Invitrogen) according to the manufacturer's instructions. Cells were grown to confluence of approximately 20–25% in a six-well plate 18–24 h prior to transfection. Transfections were done twice, once within a 24 h interval and another at 48 h. The cells were processed 72 h after the first transfection. As a control, siRNA designed to target GFP (Dharmacon) was used.

IMMUNOFLUORESCENCE

Immunostaining for p53BP1 performed on cells plated onto glass coverslips and processed for RNAi. After the transfection period, cells were washed twice with PBS, the cells were then fixed with PBS/3% paraformaldehyde for 10 min at RT. After two PBS washes, cells were permeabilized with PBS/0.5% NP40 and later blocked with PBG [PBS/0.2% fish gelatin. 0.5% bovine serum albumin (BSA)] for 30 min. Coverslips were then incubated with the rabbit anti-p53BP1 antibody (Novus NB100-304A-1), at a concentration of 1:500 in PBG overnight. Cover slips were then rinsed three times with PBG solution and incubated with secondary TRITC-conjugated goat anti-rabbit antibody (Jackson Immunoresearch) in PBG at a concentration of 1:500 for 45 min at RT. Cover slips were rinsed two times with PBG. Coverslips were then incubated with PBG and 4,6-diamidino-2-phenylindole (DAPI) at 100 ng/mL to visualize the nuclei. Coverslips were mounted on to slides with embedding media. Images were collected with an Olympus BX61 fluorescence microscope using a 60× objective connected to a Hamamatsu ORCA-ER CCD camera, controlled by the SlideBook 5.1 image capture software.

CELL CYCLE ANALYSIS BY FACS

The cells were collected and rinsed twice in cold PBS/2 mM EDTA, resuspended in 7 mL of PBS/2 mM EDTA/2% BSA, 3 mL of cold 100% Ethanol was added drop wise and the cells were kept at 4°C for 24 h fixation. The cells were then spun down and resuspended in 0.5 mL of PBS/2 mM EDTA. Ten microliters of heat inactivated RNase A (10 mg/mL) and 25 μL of Propidium Iodide (1 mg/mL) were added and the cells were incubated at 37°C for 30 min. The samples were then analyzed using a FACS Calibur Flow Cytometer.

CO-IMMUNOPRECIPITATIONS

The immunoprecipitations were performed as described in (Loayza and de Lange, 2003).

RESULTS

DEPLETION OF AJUBA BY siRNA LEADS TO S-PHASE DELAY IN HTC75 CELLS

We performed siRNA depletion of Ajuba in HTC75 cells using three different target sites. The most effective depletion was observed by Western blot with siRNA #3 (Figure 1C). In all three cases, a significant reduction in total cell count was observed, down to approximately 50% of viable cells compared to the GFP siRNA control (Figures 1A,B), at 72 h after transfection. We then sought to determine whether the cells at this time point showed a specific alteration in their cell cycle profile. To this end, cells were fixed, stained with propidium iodine and processed for FACS analysis for DNA content. In all three cultures treated with Ajuba siRNA, cells exhibited a significant increase in their number in S phase: respectively 42.3, 44.9, and 46.5% in S phase in cells treated with siRNA #1, 2, or 3, compared with 24.95% in the GFP siRNA control (Figure 2A). An average of three experiments solidified this observation, with siRNA #3 having the strongest effect, with 38% of cells in S phase versus 25.1% of the cells in the control siRNA (Figure 2C). We noted also a slight but reproducible increase in the number of cells in G2/M with siRNA #3: 17.5% versus 12.4% of the cells in the GFP siRNA control. There was also a notable increase

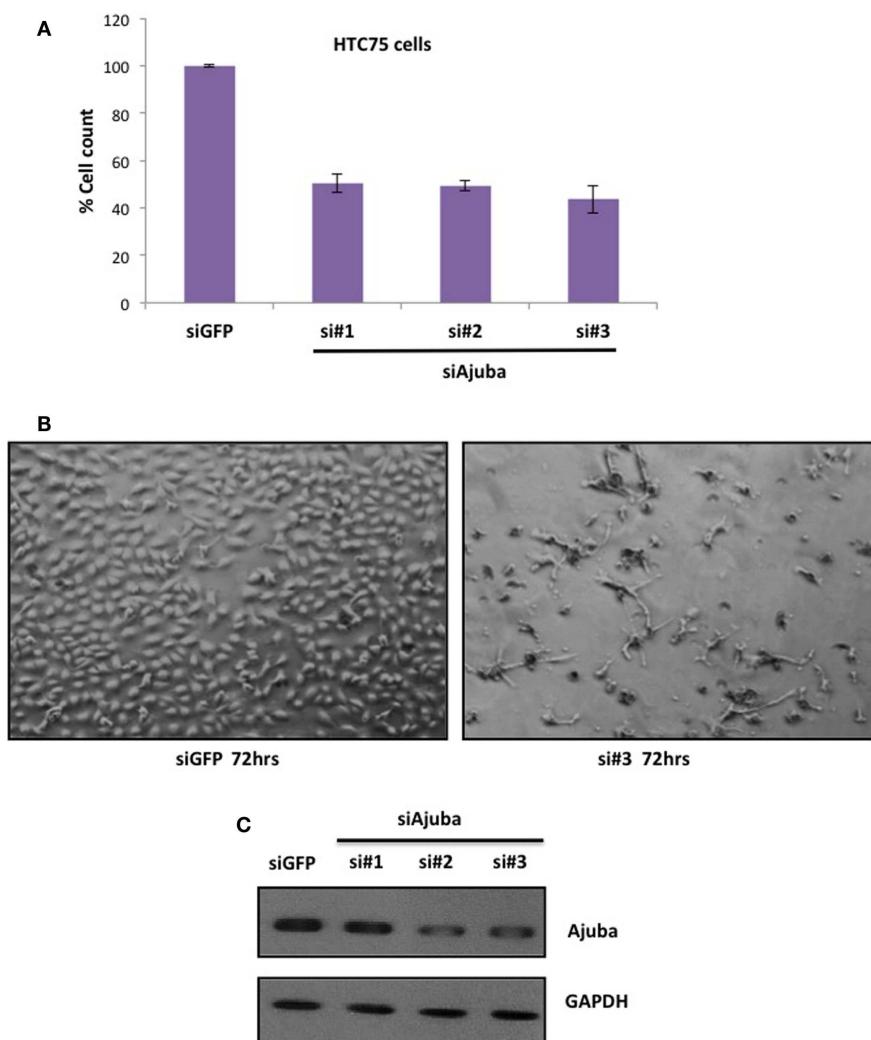


FIGURE 1 | Depletion of Ajuba in HTC75 cells results in reduced cell number. (A) Quantitation of three independent siRNA experiments with cell counts performed at 72 h after the first transfection. (B) Cells

shown 72 h after transfection, with GFP siRNA as controls. (C) Western blot showing the depletion of Ajuba by siRNA with three different target sites.

in cells with sub-G1 DNA content in all three siRNA depletions compared to controls (Figure 2A). We concluded that depletion of Ajuba led to a delay in S phase, possibly due to checkpoint activation. Protein extracts were prepared from these cells in order to probe the molecular effects of Ajuba depletion. Given the cell cycle profile observed, we were particularly interested in markers characteristic of this particular phase of the cell cycle. We found that, in the viable cells, Rb was hyperphosphorylated (Figure 2B), compatible with the cells having passed the G1/S transition. Cyclin A2 was also found at high levels (Figure 2B), a feature of cells undergoing DNA replication.

DEPLETION OF AJUBA RESULTS IN ATR ACTIVATION FOLLOWED BY APOPTOSIS IN HTC75 CELLS

The delay of cells in S phase prompted us to assess the level of endogenous DNA damage in cells depleted for Ajuba. To that end,

we stained for a known marker involved in DNA repair, p53BP1, which accumulates at sites of DNA damage early in the response. We found that depletion of Ajuba led to a significant increase of nuclei with more than five p53BP1 foci (Figures 3A,B), with 37% of nuclei compared to 2% of nuclei in GFP control experiments. In HTC75 cells, there is an average of two p53BP1 foci, which could be detected in control siRNAs and represented a background level in these cells. This apparent induction of the DDR in S phase likely activated a known DNA repair pathway, and in particular ATR, sensitive to DNA replication stress. Indeed, there was activation of ATR following Ajuba depletion, as observed by phosphorylation of Chk1 at residues Ser-345 and Ser-296, which are both ATR-dependent (Liu et al., 2000; Okita et al., 2012) (Figure 3C). In accordance with this finding, p53 was also weakly activated by Ajuba depletion (Figure 3D), as observed by Ser20 phosphorylation associated with siRNA #3. It is possible that this effect is due

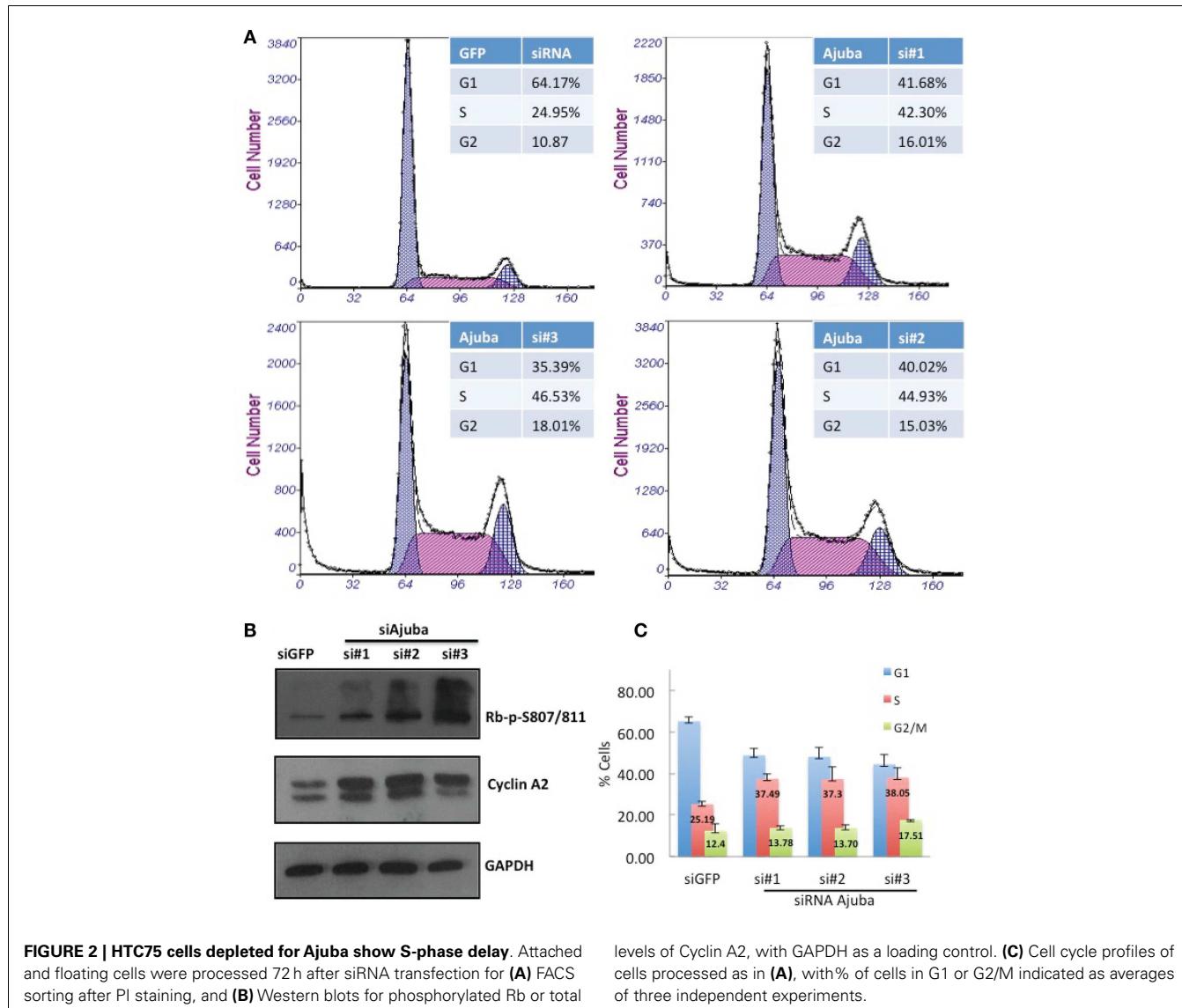


FIGURE 2 | HTC75 cells depleted for Ajuba show S-phase delay. Attached and floating cells were processed 72 h after siRNA transfection for **(A)** FACS sorting after PI staining, and **(B)** Western blots for phosphorylated Rb or total

levels of Cyclin A2, with GAPDH as a loading control. **(C)** Cell cycle profiles of cells processed as in **(A)**, with % of cells in G1 or G2/M indicated as averages of three independent experiments.

to activation of ATM as a subsequent effect, although we could not detect perceptible Chk2 phosphorylation (not shown). In all cases, the effects were again best seen with siRNA#3. Altogether, these results support the conclusion that Ajuba depletion led to phosphorylation of Chk1 and activation of p53, suggesting a role for Ajuba in repressing the ATR pathway.

We then analyzed the possible activation of apoptosis following Ajuba depletion by probing for PARP, which is cleaved by caspase 3 upon induction of apoptosis. We found extensive accumulation of the PARP cleavage product upon Ajuba depletion, again most significantly with siRNA #3 (Figure 4A). Concomitantly, the number of dead cells doubled for siRNAs #1 and 2, representing 18% of the cell count, compared to 7% with the GFP siRNA control, and topped 30% with siRNA #3 (Figure 4B).

We conclude that the cell death observed was due to the activation of the endogenous apoptotic pathway following ATR and p53 and activation.

DEPLETION OF AJUBA IN IMR90 CELLS RESULTS IN A G2/M DELAY

It was important to analyze the response to Ajuba depletion in another, unrelated cell line in order to establish the importance of the results, and also to address whether the molecular events were specific to tumor cells, or applicable to normal, non-transformed, diploid cells. We chose for this purpose the cell line IMR90, a commonly used primary human fibroblast line. We found that the effects of Ajuba depletion were strikingly similar between HTC75 and IMR90, and not acquired properties as part of a tumor phenotype.

Depletion of Ajuba in IMR90 cells (Figure 6A) led to a reduction in cell count (Figures 5A,B) and an apparent delay in the cell cycle (Figure 5C). In this case, the delay appeared to be at the G2/M phase (averages of 22.79% against 9.23% in controls) (Figure 5C), corresponding to another known checkpoint for ATR-Chk1 (through Cdc25C, see Discussion). In depleted cells, an increase in Cyclin A2 (Figure 6C), phosphorylation of

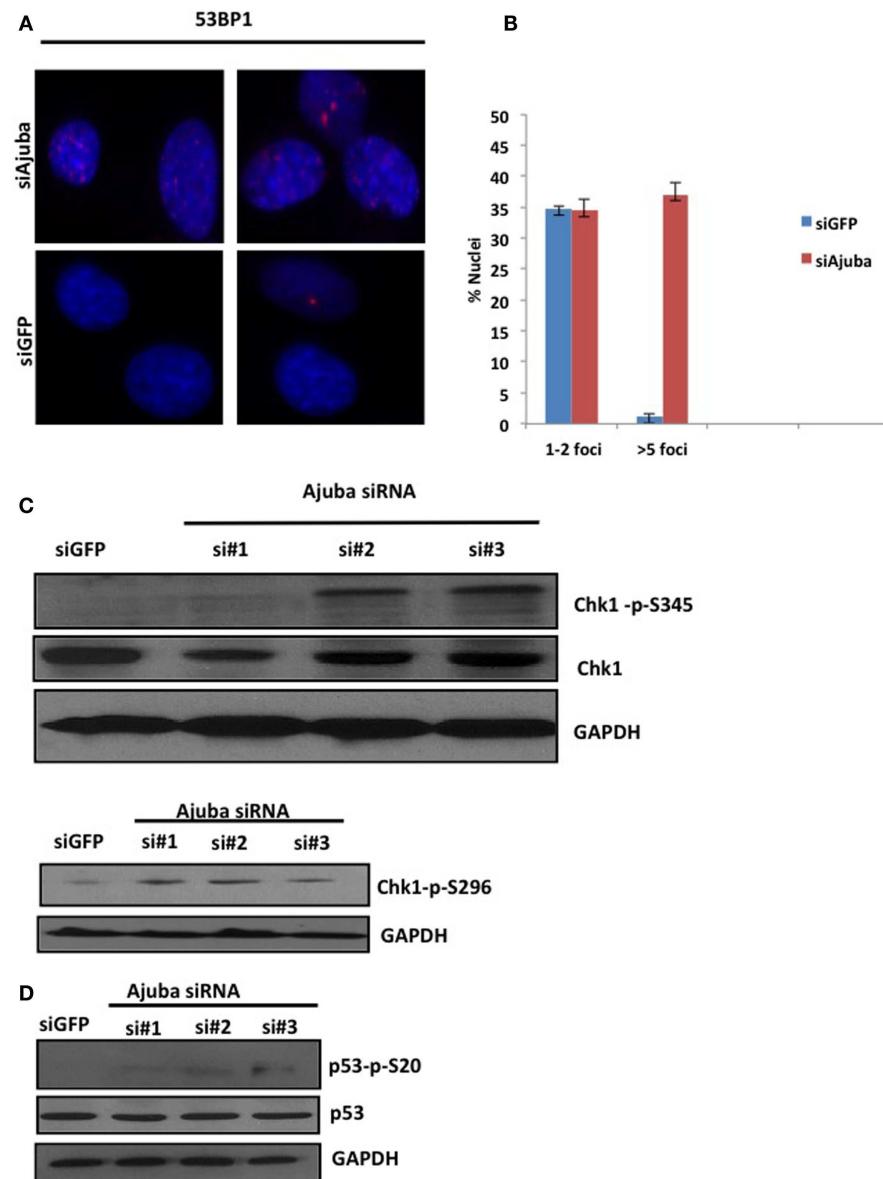


FIGURE 3 | Depletion of Ajuba results in activation of the DNA damage response. (A) Staining for p53BP1 foci on cells fixed 72 h after transfection with siRNA #3, and siGFP as a negative control. Quantification of the number of p53BP1 foci on three independent siRNA experiments shown in **(B)**. **(C, D)** Western blots for induction of Chk1 phosphorylation and induction of p53 phosphorylation, showed for the three target sites, 72 h after siRNA transfection.

Chk1 (**Figure 6D**), and, to a lesser degree, hyperphosphorylation of Rb (**Figure 6C**), were observed, most prominently with siRNA #3 (not shown). The induction of p53BP1 foci was also evident in Ajuba-depleted cells (**Figure 6B**). We did detect a low level of PARP cleavage indicating some degree of apoptosis in the cell population (**Figure 6D**), in accordance with what we observed in HTC75 cells. However, massive apoptosis was not observed nor expected, given the low degree of apoptosis activation in human fibroblasts (Duelli and Lazebnik, 2000). Thus, the nature of the response was highly similar in both cell types analyzed, with, in both cases, an obvious activation of ATR signaling.

We conclude that depletion of Ajuba in IMR90 leads to a qualitatively similar response to our tumor cell system HTC75, which corresponds to ATR activation, but with a different outcome regarding the nature of the cell cycle profile (G2/M delay in IMR90 versus S-phase delay in HTC75). This variation could be due to the different downstream effects of ATR signaling in these two different cell types.

AJUBA IS IN A COMPLEX WITH RPA IN UNPERTURBED HTC75 OR IMR90 CELLS

In order to obtain insight on the role of Ajuba in early ATR activation, we reasoned that it could inhibit signaling in the absence

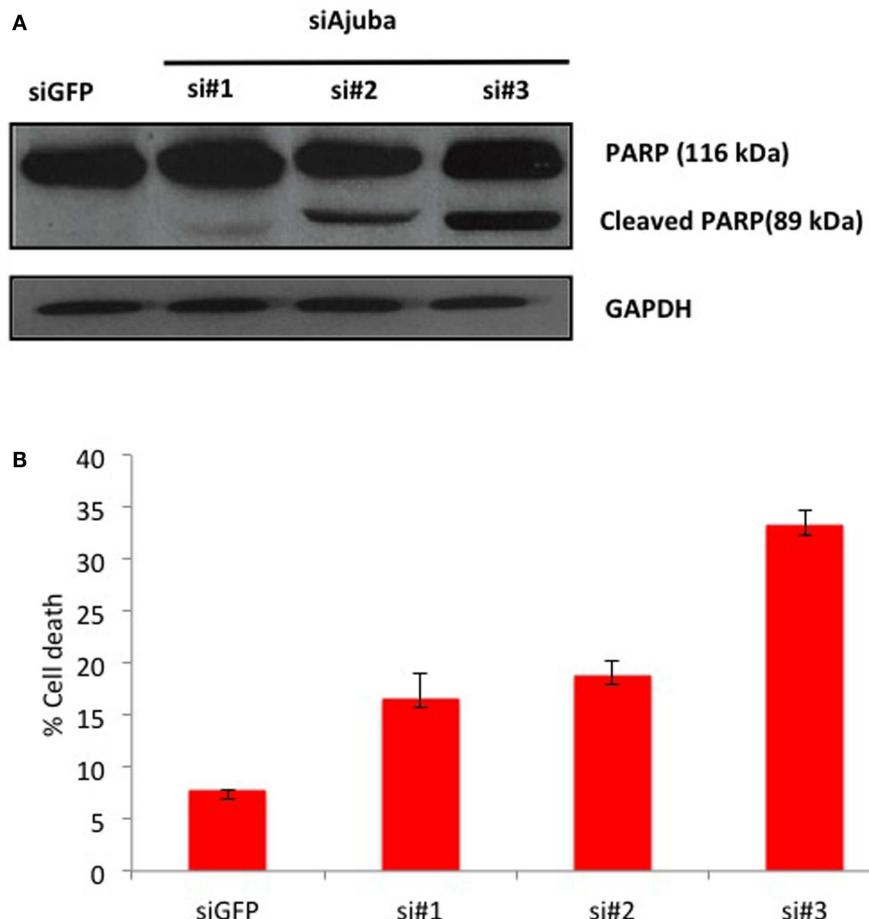


FIGURE 4 | HTC75 cells depleted for Ajuba undergo apoptosis. **(A)** Western blot probed with anti-PARP antibody (top), with GAPDH as a loading control (bottom), for the siRNAs indicated on top of the lanes. **(B)** Cell viability measured by Trypan Blue staining, 72 h after transfection with the indicated siRNAs.

of extensive DNA damage during the course of a normal S phase, and that depletion of Ajuba might engage an inappropriately massive response to replication stress. One possibility of how Ajuba exerts its influence on ATR signaling is suggested by our analysis of the role of another, related, LIM protein, TRIP6. We have found that TRIP6 binds POT1 by associating with the OB folds of the protein, and represses the DDR at telomeres (Sheppard and Loayza, 2010). Given the high similarity between TRIP6 and Ajuba, we hypothesized that the Ajuba LIM domains could be dedicated to associate with the RPA OB folds, a known platform for ATR activation (Xu et al., 2008). We tested this hypothesis by asking whether we could immunoprecipitate RPA with Ajuba antibodies (Figure 7A). We found that, in both HTC75 and IMR90 cells, RPA32 could be pulled down with a monoclonal anti-Ajuba antibody as well as with an anti-Ajuba peptide serum, suggesting an interaction between the RPA complex and Ajuba in these cells. Since RPA phosphorylation is required for ATR activation, we hypothesized that Ajuba could prevent this modification. A direct prediction of this model is that, in Ajuba-depleted cells, RPA should be detected as a phosphorylated form indicating activation of the early steps in the ATR pathway. We have tested a monoclonal

antibody raised against phosphorylated RPA32-Thr21, known to be PIKK-dependent (Anantha et al., 2007), and found that this form of RPA was significantly increased in Ajuba-depleted cells (Figure 7B). Again, this effect was observed in both cell types used in this study. It would be interesting to test other RPA phosphorylation sites (Liu et al., 2012), such as Ser33 (ATR-dependent) or Ser4/8 (DNA-PK-dependent). We propose a model (Figure 7C), based on our results, in which Ajuba, in unperturbed cells, associates with RPA and protects RPA from unscheduled phosphorylation events, which could lead to an inappropriate ATR response.

DISCUSSION

This study describes the implication of a novel partner in the DDR, the LIM protein Ajuba. We show here that Ajuba can be described as an inhibitor of the ATR-dependent DDR. This conclusion is based on our observations that depletion of Ajuba leads to a genome-wide DDR which is consistent with ATR activation, such as Chk1 phosphorylation, p53 activation, and induction of p53BP1 foci. The resulting response is a strong overall activation of the pathway as judged by the detection of PARP cleavage,

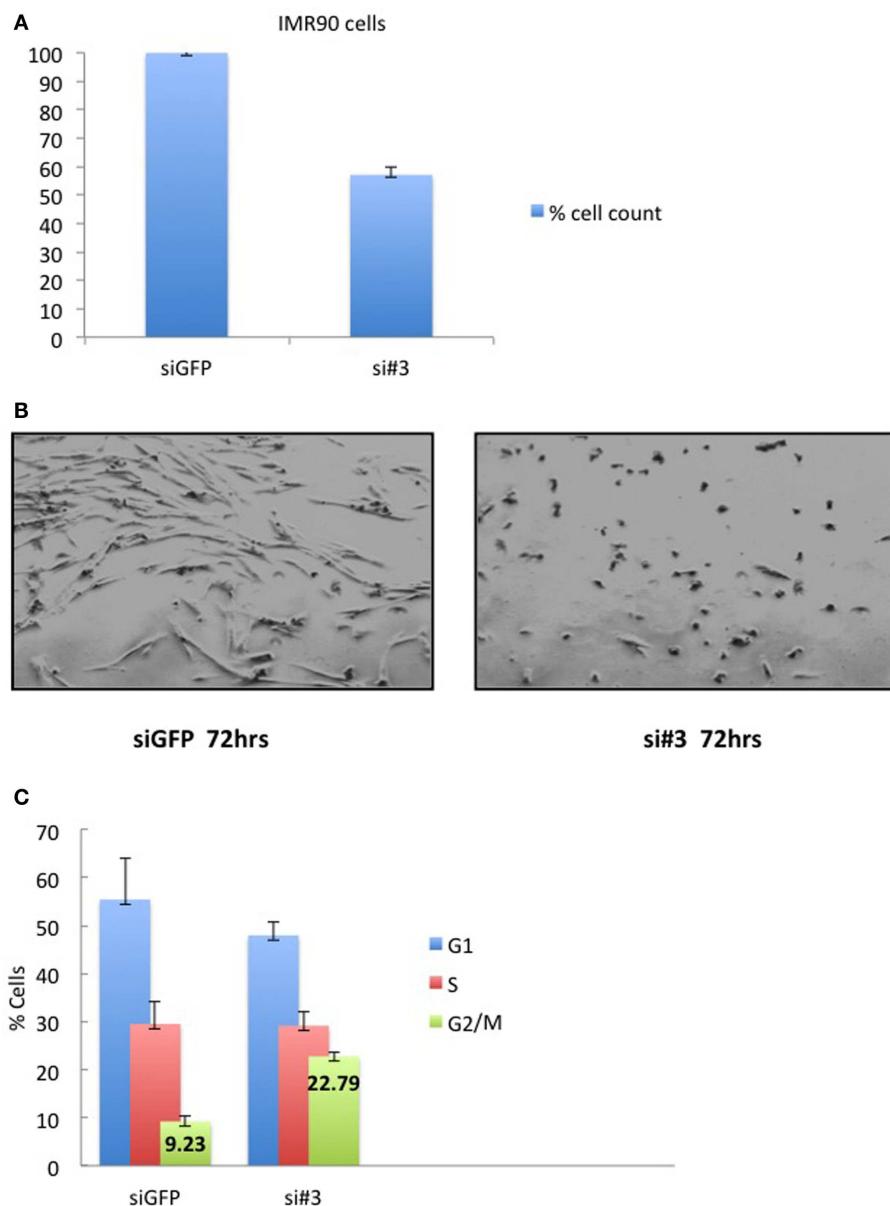


FIGURE 5 | IMR90 cells depleted for Ajuba have reduced cell number and a G2/M delay. **(A)** Cell counts of IMR90 cells taken 72 h after transfection of siRNA #3. **(B)** Picture of IMR90 cells for siRNA #3 taken

72 h after transfection. **(C)** Cell cycle profiles of cells processed as in **(B)**, with % of cells in G2/M indicated as averages of three independent experiments.

indicating the induction of an apoptotic response. Since these effects are observed in cells that are not experiencing exogenous insults, such as UV irradiation or treatment with drugs inhibiting DNA replication, we argue that Ajuba protects against an unscheduled and excessive response to endogenous DNA damage, which we believe is likely to come from spontaneous replication stress. These possible endogenous DNA damage signals could be the sites of accumulation of p53BP1, possibly representing fork collapses, misincorporated nucleotides, or intra or inter-strand crosslinks for instance. Although our experiments do not address the type of damage eliciting the response, we argue that this damage is

normally too weak to activate a full-blown DDR in the presence of Ajuba, but, in Ajuba-depleted cells, can lead to an inappropriate and unscheduled genome-wide response which is lethal to most of the cells and leads to apoptosis. Our results are compatible with other reports (Sørensen et al., 2003; Sorensen et al., 2004) that found the ATR-Chk1-Cdc25A pathway being part of a “surveillance mode” during a normal S phase, but can be activated into an “emergency” DDR after treatment with hydroxyurea, aphidicolin, or UV for instance. We propose here that Ajuba is in this context part of a system that keeps the ATR response in a “surveillance” mode, which could be relieved after extensive exogenous DNA

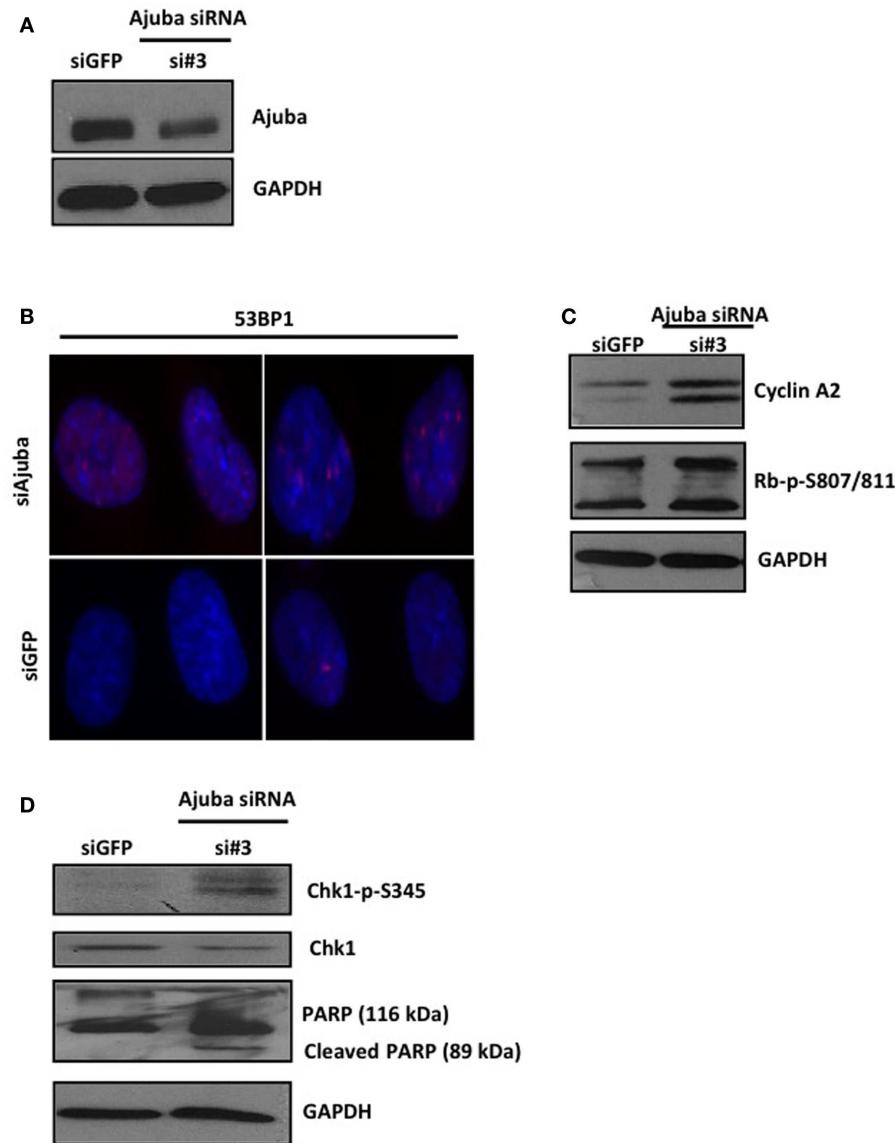


FIGURE 6 | IMR90 cells depleted for Ajuba undergo a DNA damage response. (A,C,D) Western blots for Ajuba, Cyclin A, Rb, Chk1 phosphorylation, and PARP cleavage on lysates prepared from IMR90 cells

72 h after transfection with siRNA #3. The loading controls with GAPDH for each blot are shown. (B) Staining for p53BP1 in Ajuba-depleted cells (siRNA#3), with siGFP as a control.

damage. Thus, Ajuba-depleted cells would respond with excessive strength to endogenous and sporadic DNA replication lesions.

We hypothesized that Ajuba plays an important role in inhibiting the DDR mostly in cells of tumor origin due to rampant genome instability and high chromosomal DNA damage in these cells. We therefore analyzed the response in normal human diploid fibroblasts to ask whether the role of Ajuba was important in a context of low level of endogenous DNA damage. Our results show that indeed Ajuba is also important in non-tumor cells to repress the ATR response in absence of exogenous DNA damage. In the diploid fibroblasts we used, the cells responded by a delay in the cell cycle and cell death as well. We observed a qualitative difference in the nature of the cell cycle delay, which corresponded to a G2/M

delay in IMR90 cells compared with a S-phase delay in HTC75 cells. The difference in this aspect of the response could be due to the intrinsic wiring of the ATR response in normal fibroblasts, leading to a robust inhibition of Cdc25C and delay of entry into mitosis in these cells. Tumor cells, however, could experience an effect mostly on Cdc25A, which is degraded after Chk1 activation (for review, see Zhou and Bartek, 2004). Degradation of Cdc25A has multiple effects in various parts of the cell cycle, one of them being a strong delay in S phase. More work is required to establish which of Cdc25A or Cdc25C is mostly impacting the cell cycle profiles in either cell types, as well as in other cell types. We think it plausible, although not addressed here, that in both cases the signaling is initiated in S phase in response to DNA replication

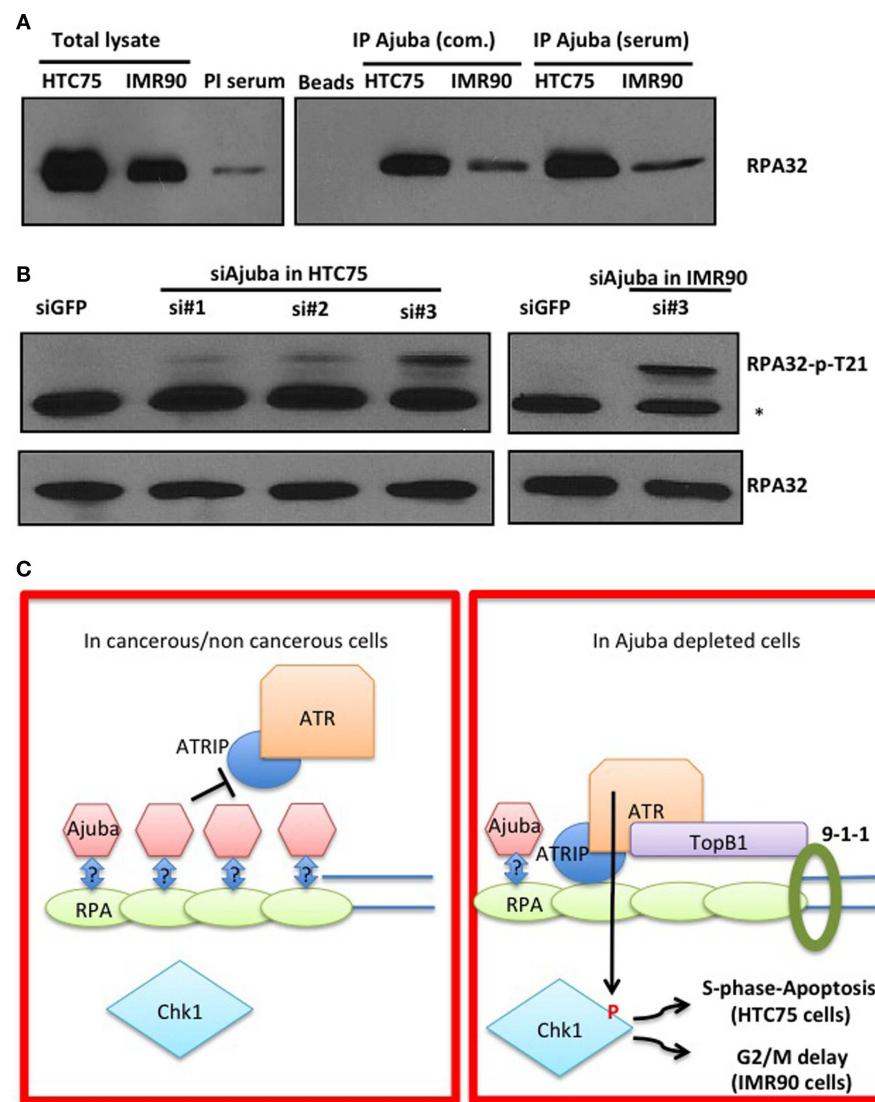


FIGURE 7 | Ajuba associates with RPA in HTC75 and IMR90 cells. (A) IP-Western probed with a total anti-RPA32 antibody after immunoprecipitation with a commercial anti-Ajuba antibody (com.) or anti-peptide serum from IMR90 or HTC75 extracts as indicated. Left panel: total lysates (input) for each cell line, and the pre-immune serum (PI) used as a control for the

immunoprecipitation. **(B)** Western for RPA32-p-Thr21 in both HTC75 and IMR90 Ajuba-depleted cells. A non-specific band is marked by a *. Total RPA32 levels are shown at the bottom. **(C)** Model for the role of Ajuba in repression of ATR. The "?" indicates that the interaction between Ajuba and RPA could be direct or indirect. See text for details.

stress. Thus, the role of Ajuba appears to be important in the context of a normal S phase. In both cell types analyzed here, the response observed is so extensive as to lead to cell death and apoptosis, particularly in the case of the HTC75 cells. Also, this aspect of the response, including activation of p53 and cleavage of PARP, could be under dependence of ATM, known to be activated by a sustained ATR response, and not necessarily a direct effect of the ATR-Chk1 pathway. Such effects have been noted by others (see Hurley and Bunz, 2007).

Overall, these observations imply that ATR is poised for a full-blown response to DNA damage and the pathway requires repression, exerted in part by Ajuba, for progression through the cell cycle, which would allow for local ATR activation at sporadic

sites of replication stress such as replication fork collapse or excessive production of single stranded DNA, for instance. Repressors such as Ajuba would keep the response localized and allow for rapid repair and continuation of S phase.

Following the observations linking Ajuba to the repression of the ATR response, we sought to determine the mechanism of action of Ajuba in the ATR activation pathway. We focused on RPA, an essential single strand DNA binding protein constituted by three OB-fold-containing subunits, RPA70, RPA32, and RPA14. RPA has long been documented as playing essential roles in DNA replication, DNA repair and recombination (Wold, 1997), and is an early player in ATR activation following DNA damage (Zou and Elledge, 2003). RPA70 constitutes a

platform for the binding of a number of proteins essential for ATR activation, through direct contacts with ATRIP and RAD9, and further recruiting ATR and TOPBP1 (Xu et al., 2008). It is tempting to speculate that Ajuba could bind RPA and prevent ATR activation in undamaged or unstressed cells, thereby preventing the formation of ATR-activating foci. In addition, our laboratory has discovered a similar interaction between POT1, the telomeric overhang binding protein, and the LIM protein TRIP6 (Sheppard and Loayza, 2010). This interaction was initially discovered through a yeast two-hybrid screen, and involved the POT1 N-terminal OB folds and the C-terminal LIM domains of TRIP6. We hypothesized that this domain interaction could be conserved in other protein partners, and in particular between OB-fold-containing RPA and LIM-containing Ajuba. Our results indeed support this hypothesis since RPA could be found in a complex with Ajuba. We are currently following up on this result in asking whether there is a direct interaction between the OB-folds found in RPA and Ajuba in a recombinant protein system. A clear prediction of our model of RPA shielding by Ajuba (Figure 7C) is that, in Ajuba-depleted cells, we should observe increased RPA32 phosphorylation, an early step in ATR activation. Supporting this model, we could clearly detect such an event by looking at RPA32-Thr21 phosphorylation, but more work is required to dissect the exact role of Ajuba in this process. Indeed, the phosphorylation of RPA32 displays a complex pattern, with Thr21 being dependent on ATR itself, Ser33 on ATM and believed to occur during a sustained response, and Ser4 and Ser8 believed to be essential for the early phase of the induction, perhaps even before activation of ATR itself, with DNA-PK as an effector kinase (Liu et al., 2012). Our results show that

Ajuba protects from unscheduled Thr21 phosphorylation, definitely placing this molecule at the level of RPA in the repression of the ATR response. Our working model given our results is that Ajuba interacts with the RPA complex and prevents inappropriate phosphorylation of RPA32. It will be interesting to address in the future whether such a role is restricted to S phase or important throughout the cell cycle. Also, whether Ajuba prevents DNA-PK-dependent, or ATR-dependent phosphorylation, or both, remains to be established.

This leaves an important question: how can ATR be activated in the course of DNA replication stress or DNA damage? Possibly, free RPA exists in the cell that could get phosphorylated following such lesions, modifications that could significantly reduce the binding affinity for Ajuba and generate a free, unbound pool able to generate a local ATR response, or a more sustained one depending on the extent of the damage. We are currently testing with recombinant proteins whether the interactions between Ajuba and RPA are direct, and whether specific phosphorylation sites reduce the binding affinities of these interactions, as we would predict. A broader impact of Ajuba and related molecules is that they could have oncogenic properties during early events of cellular transformation by inhibiting the protective or tumor suppressive effects of ATR.

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Hold your horSSEs: controlling structure-selective endonucleases MUS81 and Yen1/GEN1

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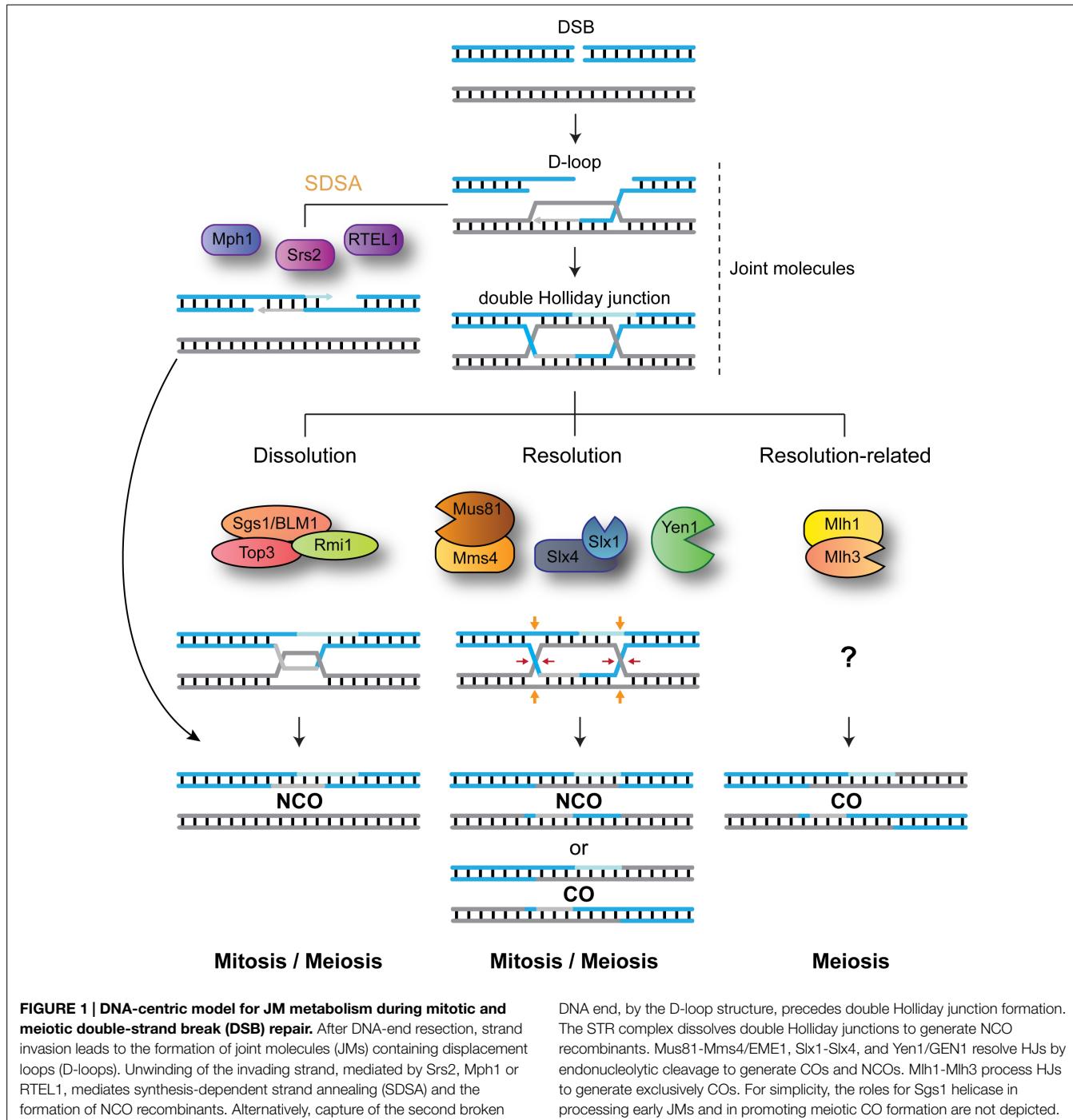
Repair of DNA lesions through homologous recombination promotes the establishment of stable chromosomal interactions. Multiple helicases, topoisomerases and structure-selective endonucleases (SSEs) act upon recombining joint molecules (JMs) to disengage chromosomal connections and safeguard chromosome segregation. Recent studies on two conserved SSEs – MUS81 and Yen1/GEN1 – uncovered multiple layers of regulation that operate to carefully tailor JM-processing according to specific cellular needs. Temporal restriction of SSE function imposes a hierarchy in pathway usage that ensures efficient JM-processing while minimizing reciprocal exchanges between the recombining DNAs. Whereas a conserved strategy of fine-tuning SSE functions exists in different model systems, the precise molecular mechanisms to implement it appear to be significantly different. Here, we summarize the current knowledge on the cellular switches that are in place to control MUS81 and Yen1/GEN1 functions.

Keywords: nuclease, DNA repair, recombination, replication, Holliday junction, Cdk, Cdc5/PLK1, Cdc14

Establishment and Safe Removal of DNA Joint Molecules During Recombinational DNA Repair

In all organisms, the preservation of hereditary information relies on repair mechanisms that counteract the lesions constantly inflicted on their DNA. Cells have matched the diversity and complexity of these injuries with a staggering assortment of DNA repair pathways specialized in specific types of damage. While insults like chemical modifications of the nucleotide bases and single-strand breaks are some of the most abundant (Lindahl and Barnes, 2000), DNA double-strand breaks (DSBs) pose a higher risk for the cell, as failure to repair them may lead to the loss of whole chromosomal arms.

Homologous recombination (HR) is an evolutionarily conserved DSB repair pathway that resorts to an intact DNA molecule with an identical (or nearly identical) sequence, such as the sister chromatid or the homologous chromosome, to restore the integrity of the broken strands. For this purpose, the HR machinery drives the damaged DNA duplex through a series of molecular exercises that include DNA end-resection, homology search, strand invasion and DNA synthesis to retrieve the missing information (Heyer et al., 2010; Symington and Gautier, 2011). One central feature of HR is that pairing and strand exchange reactions lead to the formation of increasingly stable recombination intermediates. At the chromosomal level, these structures translate into inter-sister (or inter-homolog) DNA joint molecules (JMs) that must be disconnected prior to cell division. To solve this problem, cells frequently employ anti-recombinogenic helicases that dislodge the



invading DNA strand on displacement loop structures (D-loops; **Figure 1**). However, long-lived D-loops may occasionally capture the second broken end, which then primes DNA synthesis using the displaced strand as a template. Sealing of the nicks at the end of the newly synthesized strands leads to the establishment of fully ligated four-way junctions, termed Holliday junctions (HJs; Liu and West, 2004; Holliday, 2007). Due to the covalent nature of the link that is formed as they mature, HJs are arguably the most dangerous of all recombination intermediates that contribute to

the linkage of two DNA duplexes. It is important to point out that such potentially dangerous intermediates appear not only as a consequence of DSB repair, but also during DNA replication (Giannattasio et al., 2014), since HR is also involved in both replication fork reactivation and post-replicative ssDNA gap-filling (Branzei and Foiani, 2010; Rass, 2013).

Despite the risks of interlocking the two recombining chromosomes, the formation of JMs is crucial for HR repair both during mitosis and meiosis. Maturation of early recombination

intermediates into HJs within meiotic JMs precedes the formation of crossovers (COs), repair products characterized by the physical exchange of the DNA duplexes flanking the branch point (Schwacha and Kleckner, 1995; Allers and Lichten, 2001; Petronczki et al., 2003). Importantly, COs not only result in the reassortment of genetic information between maternal and paternal genomes, but are also required for the correct bipolar segregation of homologs during the first meiotic division (Schwacha and Kleckner, 1995; Allers and Lichten, 2001; Petronczki et al., 2003). Since inter-homolog exchanges can lead to loss-of-heterozygosity (LOH), mitotic cells disengage most JMs at an early stage to prevent CO formation (Ira et al., 2003; Bzymek et al., 2010). In addition, during the mitotic cell cycle, removal of late JMs that contain HJs is biased toward pathways that promote formation on non-crossover recombinants (NCOs; Dayani et al., 2011).

So how do cells modify JM-processing according to the specialized cellular needs of mitosis and meiosis? In order to efficiently disengage recombination intermediates, while having flexibility toward the choice of recombination outcome (CO vs. NCO), cells have evolved a blend of DNA-processing enzymes with specialized abilities (Figure 1). Helicases, such as Srs2, Mph1/FANCM, or RTEL1, are capable of unwinding early recombination intermediates like D-loop structures to generate exclusively NCO recombinants (Ira et al., 2003; Barber et al., 2008; Prakash et al., 2009). Structure-selective endonucleases (SSEs), such as MUS81-EME1 (Mus81-Mms4 in *Saccharomyces cerevisiae*; Mus81-Eme1 in *Schizosaccharomyces pombe*; hereinafter, we will use the term MUS81* to refer to all these orthologs collectively), SLX1-SLX4 (Slx1-Slx4 in *S. cerevisiae* and *S. pombe*) and GEN1 (Yen1 in *S. cerevisiae*; absent in *S. pombe*), can cleave late recombination intermediates, containing HJs or HJ precursors, to generate a mixture of COs and NCOs (Boddy et al., 2000, 2001; Kaliraman et al., 2001; Fricke and Brill, 2003; Ip et al., 2008; Andersen et al., 2009; Fekairi et al., 2009; Munoz et al., 2009; Svendsen et al., 2009; Schwartz and Heyer, 2011). The STR complex (BTR in humans), composed of the RecQ helicase Sgs1 (BLM), the topoisomerase Top3 (TOP3 α), and Rmi1 (RMI1/2), promotes the convergent branch-migration and decatenation of double HJs to generate NCOs (Gangloff et al., 1994; Fabre et al., 2002; Wu and Hickson, 2003). Finally, the Mlh1-Mlh3 nuclease mediates HJ processing to generate exclusively COs through a mechanism that remains elusive (Zakharyevich et al., 2012; Ranjha et al., 2014; Rogacheva et al., 2014; Figure 1).

Despite the identification of specialized pathways in JM-processing, most (perhaps all) JM-processing enzymes are expressed and function during mitosis and meiosis. Therefore, one key question that arises and remains largely unanswered is: how do cells tailor pathway usage to satisfy their specialized needs? Recent studies focusing on MUS81* and Yen1/GEN1, two SSEs with important roles in mitotic and meiotic DNA repair, have started to unveil the subtle manipulations that cells utilize to tame their potentially deleterious activities, blocking or unleashing them according to their particular requirements. In the next sections, we will attempt to summarize the current knowledge on the mechanisms employed to control SSE function.

Regulation of MUS81* and Yen1/GEN1 Structure-Selective Nucleases

“Edged tools are dangerous things to handle, and not infrequently do much hurt”

– Agnes Repplier (1855–1950)

The MUS81* Nucleases

MUS81* belongs to the XPF/Rad2 family of nucleases, whose structural and functional features have been superbly reviewed elsewhere (Ciccia et al., 2008). Therefore, we will only briefly highlight some of its most relevant characteristics for our topic. Like all the other members of the family, MUS81* exists as a heterodimeric protein complex and harbors the distinctive ERCC4 nuclease domain, in addition to helix-hairpin-helix (HhH) motifs in both the N-terminal and C-terminal regions (Figure 2). Its partner proteins (Mms4 in budding yeast, Eme1 in fission yeast, EME1 and EME2 in human cells) have a similar domain organization, with exception of the absence of the N-terminal HhH motif. Despite being indispensable for MUS81* stability and the nuclease activity of the complex, Mms4, Eme1, EME1, and EME2 are regarded as non-catalytic subunits because they contain mutations in key residues of the ERCC4 domain.

In *S. cerevisiae*, *mms4* mutants were initially described by their increased sensitivity to the alkylating agent methyl methanesulfonate (Xiao et al., 1998) and Mus81 was isolated as a specific interactor of Rad54 in a yeast two-hybrid screen (Interthal and Heyer, 2000). Both genes were also recovered in a synthetic lethality screen of *sgs1* Δ mutants (Mullen et al., 2001). In *S. pombe*, Mus81 was identified through a yeast two-hybrid approach as an interactor of the checkpoint kinase Cds1 and found to exist in a complex with Eme1 (Boddy et al., 2000, 2001). Due to its high conservation, bioinformatic analyses succeeded in recognizing Mus81 orthologs in other organisms, including humans (Chen et al., 2001). The strong mitotic and meiotic phenotypes of *mus81* Δ and *eme1* Δ /*mms4* Δ mutants, including impaired DNA-damage repair, reduced spore viability and crossover formation led to the proposal that the MUS81* nucleases were the eukaryotic HJ resolvases (Boddy et al., 2000, 2001; de los Santos et al., 2001, 2003; Mullen et al., 2001). However, this view was controversial since the biochemical properties of these nucleases suggested a different resolution mechanism from the well-established bacterial resolvase RuvC, a homodimeric protein that introduces two symmetrical nicks in strands of like polarity across one axis of the HJ, yielding nicked DNA duplexes that can be ligated without the need of further processing (West, 1997; Haber and Heyer, 2001; Heyer et al., 2003; Heyer, 2004; Hollingsworth and Brill, 2004). MUS81* complexes from different organisms can cleave a number of different branched structures efficiently, including 3'-flaps, D-loops, model replication forks and nicked HJs, while intact HJs are generally poor substrates for this nuclease (Boddy et al., 2001; Kaliraman et al., 2001; Constantinou et al., 2002; Doe et al., 2002; Ciccia et al., 2003; Gaillard et al., 2003; Ogrunc and Sancar, 2003; Osman et al., 2003; Fricke et al., 2005; Gaskell et al., 2007; Ehmsen and Heyer, 2008; Schwartz et al., 2012; Pepe and West, 2014b). Given the broad spectrum of branched structures that the MUS81

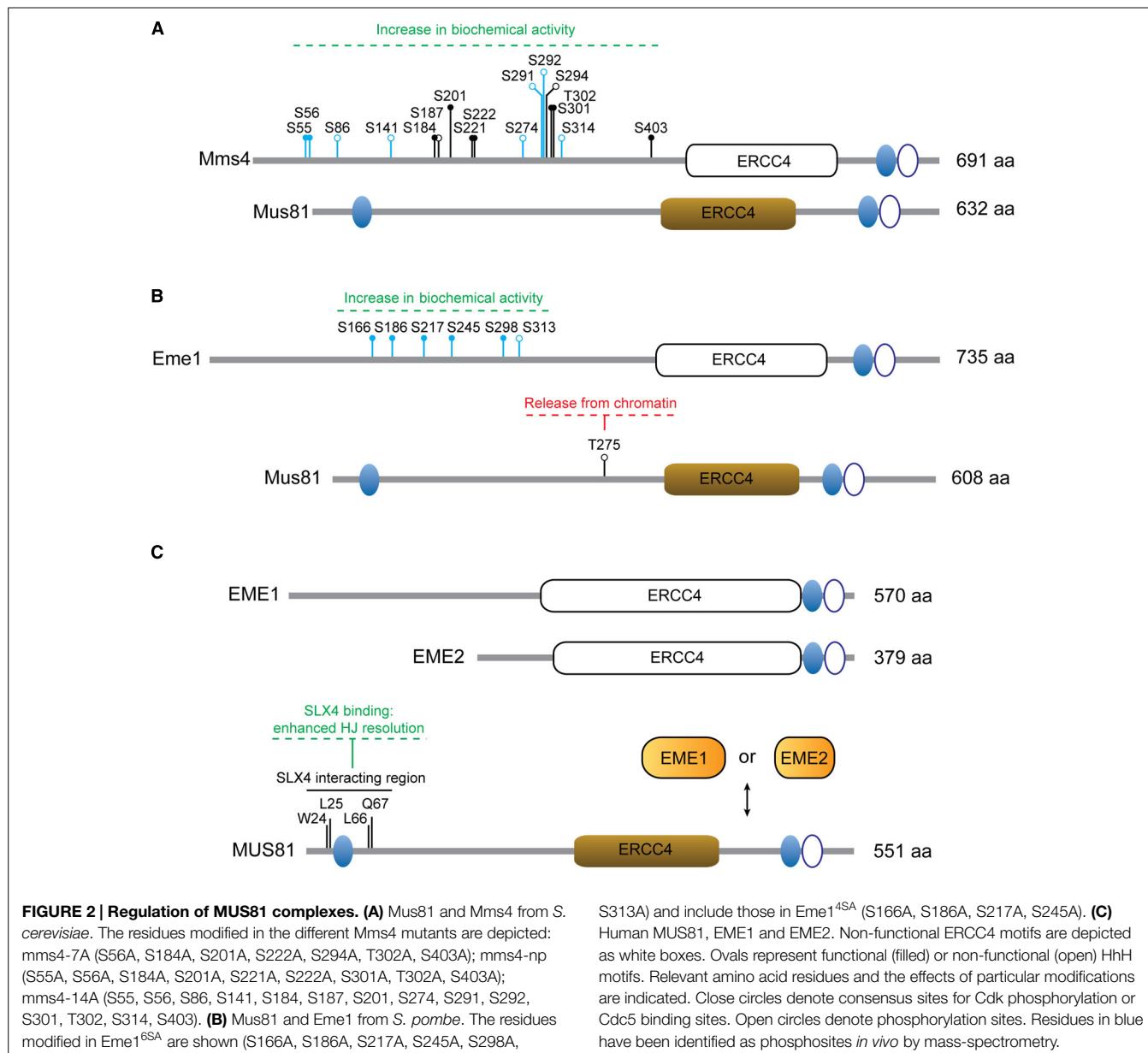


FIGURE 2 | Regulation of MUS81 complexes. (A) Mus81 and Mms4 from *S. cerevisiae*. The residues modified in the different Mms4 mutants are depicted: mms4-7A (S56A, S184A, S201A, S222A, S294A, T302A, S403A); mms4-np (S55A, S56A, S184A, S201A, S221A, S222A, S301A, T302A, S403A); mms4-14A (S55, S56, S86, S141, S184, S187, S201, S274, S291, S292, S301, T302, S314, S403). **(B)** Mus81 and Eme1 from *S. pombe*. The residues modified in Eme1^{6SA} are shown (S166A, S186A, S217A, S245A, S298A,

S313A) and include those in Eme1^{4SA} (S166A, S186A, S217A, S245A). **(C)** Human MUS81, EME1 and EME2. Non-functional ERCC4 motifs are depicted as white boxes. Ovals represent functional (filled) or non-functional (open) HhH motifs. Relevant amino acid residues and the effects of particular modifications are indicated. Close circles denote consensus sites for Cdk phosphorylation or Cdc5 binding sites. Open circles denote phosphorylation sites. Residues in blue have been identified as phosphosites *in vivo* by mass-spectrometry.

complexes can target, it was soon proposed that without strict regulation its activity might not be beneficial and give rise to potentially deleterious events (Kai et al., 2005). Interestingly, recent work from different model organisms indicates that the biological roles of the MUS81 nucleases are carefully modulated by post-translational modifications. This allows cells to tailor MUS81 function according to specific cellular needs, such as boosting its ability to process JMs that have persisted until the mitotic stage, while avoiding the unscheduled processing of other physiologically important branched DNA structures.

S. cerevisiae Mus81-Mms4

In budding yeast, Mus81 is associated to the non-catalytic subunit Mms4, with their main interaction domain residing in the C-terminal region of both proteins (Fu and Xiao, 2003). It has been

found that the biochemical activity of Mus81-Mms4 fluctuates throughout the cell cycle both in meiotic and mitotic cells, from a minimum in G1/S to a maximum at G2/M (Matos et al., 2011; Matos and West, 2014). As cells approach M-phase, Mms4 is increasingly phosphorylated, with a concomitant boost in the catalytic activity of the complex (Matos et al., 2011, 2013; Gallo-Fernandez et al., 2012; Szakal and Branzei, 2013). When the hyperphosphorylated Mus81-Mms4 from cells in G2/M is dephosphorylated *in vitro*, its nuclease activity decreases to a basal level, indicating that biochemical hyperactivation is a direct consequence of phosphorylation (Matos et al., 2011).

Two cell-cycle kinases have been implicated in both events: the Polo kinase Cdc5 and M-phase Cdc28/Cdk (Matos et al., 2011, 2013; Gallo-Fernandez et al., 2012; Szakal and Branzei, 2013). Whereas both kinase activities are required for Mus81-Mms4

activation at the G2/M transition, Cdc5 activity seems to be especially relevant as Cdc5 overexpression is sufficient to drive phospho-activation outside M-phase (Matos et al., 2011, 2013). Furthermore, Cdc5 kinase is sufficient to hyperactivate Mus81-Mms4 *in vitro* (Schwartz et al., 2012). However, the precise contributions of each of these kinases to Mus81-Mms4 regulation *in vivo* remain to be determined: is Cdk-mediated phosphorylation important only in priming Mms4 for Cdc5 binding, or does it also have a more direct role in the regulation of nuclease activity?

The generation of Mms4 mutants in which Cdk/Cdc5-dependent modification is impaired has helped us understand the biological relevance of Mus81-Mms4 phosphorylation (Figure 2A). In this sense, the *mms4-7A* (Szakal and Branzei, 2013) and *mms4-np* (Gallo-Fernandez et al., 2012) alleles were created to encode substitutions of serines or threonines at predicted Cdk (S/T-P) or Cdc5-docking (S-pS/pT-P) consensus sites in the sequence of *MMS4*. An additional mutant, *mms4-14A*, was engineered to prevent phosphorylation in both predicted and *in vivo*-validated phosphoresidues found in Mms4 from nocodazole-arrested cells (Matos et al., 2011). As expected, all three mutants are largely resistant to mitosis-specific phosphorylation (Matos et al., 2011, 2013; Gallo-Fernandez et al., 2012; Szakal and Branzei, 2013). To assess if modification of Mms4 could influence the catalytic properties of Mus81-Mms4, the nuclease activities of Mus81-mms4-np and Mus81-mms4-14A were measured in immunoprecipitates from synchronous cells at different stages of the cell cycle. Both mutants displayed impaired nuclease activation at the G2/M transition, consistent with the idea that Cdc28/Cdk and Cdc5-mediated Mms4 modification is required for Mus81 hyperactivation (Matos et al., 2011; Gallo-Fernandez et al., 2012). Although it has not been formally tested, it is expectable that *mms4-7A* will manifest similar properties.

The phenotypic analysis of *mms4-7A* and *mms4-14A* mutant strains revealed a strong sensitivity to DNA-damaging agents and a severe synthetic growth defect when combined with *sgs1Δ* (Matos et al., 2011, 2013; Szakal and Branzei, 2013). Both phenotypes are also shared with *cdc5-2* mutants, which are unable to phosphorylate and activate Mus81-Mms4 during mitosis (Matos et al., 2013). This is in contrast to *mms4-np* mutants, which show considerably milder phenotypes and only display increased sensitivity to genotoxic agents in the absence of *sgs1Δ* (Gallo-Fernandez et al., 2012). It is yet unclear why the *mms4-np*, the mutant with an intermediate number of alanine substitutions -nine-, has a milder phenotype than both the *mms4-7A* and *mms4-14A* alleles, but it has been proposed that differences in the genetic backgrounds employed by each group may account for this fact (Szakal and Branzei, 2013). Altogether, these results indicate that hyperactivation of the Mus81-Mms4 nuclease at G2/M is important for the elimination of those recombination intermediates that escape the action of Sgs1.

From a mechanistic point of view, how the phosphorylation of the nuclease drives its hyperactivation is not yet understood. It has been proposed that Mus81-Mms4 may exist in dimeric as well as tetrameric states in solution (Gaskell et al., 2007). This hypothesis would provide an intuitive and elegant system for its

hyperactivation, particularly in HJ processing, which requires a double incision for resolution of the X-shaped structure. However, *in vitro*-phosphorylation experiments with purified Mus81-Mms4 and Cdc5, followed by size-exclusion chromatography, have ruled out that the phosphorylation-dependent hyperactivation is a result of multimerization of the nuclease (Schwartz et al., 2012). An alternative possibility is that phosphorylation may lead to changes in the stability of the complex. This seems unlikely, though, as most of the mapped and predicted phosphorylation sites lie outside the interaction domain between Mms4 and Mus81, and *mms4-14A* seems to associate normally with Mus81 (Matos et al., 2011). Finally, phosphorylation events could trigger a structural change that favors binding and/or turnover of the enzyme-substrate complex. Interestingly, two phosphomimetic mutants have been proposed to represent constitutively active versions of the Mus81-Mms4 nuclease (*mms4-56E* and *mms4-56E, 184D*), as their expression promotes increased CO formation and reduced accumulation of X-shaped molecules in *sgs1Δ* mutants (Szakal and Branzei, 2013). In the future, analysis of the biochemical properties of such mutants may contribute to our understanding of the mechanism of Mus81-Mms4 nuclease activation.

At the level of protein–protein interactions, it has been reported that the binding of the N-terminal region of Mus81 and the C-terminal region of Rad27/FEN1 results in their mutual enzymatic stimulation (Kang et al., 2010; Thu et al., 2015). In addition, while the human orthologs of the Mus81-Mms4 and Slx1-Slx4 can physically interact (see Human MUS81-EME1/EME2), the initial results in budding yeast showed that these complexes could not associate nor stimulate each other *in vitro*, at least for the cleavage of different HJ substrates and model replication forks (Schwartz et al., 2012). However, more recent work has shown that Slx1-Slx4 can stimulate Mus81-Mms4 activity on 3'-flap structures (Thu et al., 2015). Furthermore, phosphorylation of the Mus81-Mms4 by Cdc5 leads to its association with the scaffold protein Dpb11 at G2/M, which can also interact with Slx4 (Gritenaite et al., 2014). While it has not been demonstrated that the formation of this Mus81-Mms4-Dpb11-Slx4 complex alters the biochemical properties of Mus81-Mms4, it may provide a system for substrate targeting, rendering Mus81 a more efficient nuclease *in vivo*.

Altogether, the emerging picture is that cell cycle stage-specific phosphorylation events are likely to modulate Mus81-Mms4 function through several complementary mechanisms: (1) direct enhancement of nuclease activity; (2) regulation of nuclease activity through stimulatory protein–protein interactions; (3) regulation of protein–protein interactions that facilitate recruitment to cognate substrates.

S. pombe Mus81-Eme1

The Mus81-Eme1 complex from fission yeast was the first eukaryotic nuclease to be considered a nuclear HJ resolvase (Boddy et al., 2001). Interestingly, the initial description of Mus81 as an interactor of the checkpoint kinase Cds1 (Rad53, CHK2) also revealed that Mus81 is indeed modified by Cds1 after hydroxyurea (HU) treatment (Boddy et al., 2000). The phosphorylation of Mus81 at T275 (T239 in the original manuscript) is required for

its association with the phosphopeptide-binding FHA domain in Cds1 (Kai et al., 2005). In turn, binding to Cds1 is a prerequisite for Mus81 hyperphosphorylation, which induces its dissociation from chromatin without affecting nuclease activity (**Figure 2B**; Kai et al., 2005). Thus, the checkpoint-mediated modification of Mus81 is thought to prevent Mus81-dependent cleavage of replication/recombination intermediates generated after HU treatment (Kai et al., 2005).

Recent work has revealed a new layer of complexity in the regulation of Mus81-Eme1. Eme1 is phosphorylated in a Rad3 (ATR)- and Chk1 (CHK1)-dependent manner, both after treatment with genotoxic agents and in the absence of Rqh1 (Sgs1/BLM). Interestingly, the modification of Eme1 is Cds1-independent and causes a marked increase in the activity of Mus81 nuclease (Dehe et al., 2013). Moreover, Eme1 is also a substrate of the cyclin-dependent kinase Cdc2 (CDK), which modifies Eme1 in a cell cycle stage-specific manner (Dehe et al., 2013).

A comparative analysis of two Eme1 mutants, one refractory to phosphorylation events in response to camptothecin (CPT) treatment (Eme1^{6SA}) and another carrying mutations in a subset of four Cdc2-consensus sites within Eme1^{6SA} (Eme1^{4SA}; **Figure 2B**), revealed identical consequences to Mus81-Eme1 phospho-activation: neither Eme1^{6SA} nor Eme1^{4SA} could be phosphorylated or biochemically hyperactivated by CPT-treatment. Consequently, the authors have suggested that Cdc2-dependent phosphorylation is required as a priming event for the subsequent CPT-induced modification (Dehe et al., 2013).

Recent results have shown that both the intra-S and DNA-damage checkpoints are blind to the presence of the type of recombination intermediates that require Mus81-Eme1 for resolution at the onset of mitosis (Mohebi et al., 2015). Therefore, future analyses of Mus81-Eme1 regulation will be essential to unravel the intricate interconnections and relative contributions of the cell cycle and the checkpoint machineries for Mus81-Eme1 activation. This is particularly relevant given the stark contrast with the situation in budding yeast, where both physical and genetic evidence have ruled out that the DNA-damage checkpoint kinases like Mec1 (ATR) or Tel1 (ATM) contribute significantly to either the phosphorylation of the Mus81-Mms4 nuclease or the ensuing resolution of late recombination intermediates (Szakal and Branzei, 2013).

Human MUS81-EME1/EME2

Homology-based searches using *S. pombe* Eme1 as a bait revealed the existence of two partners for MUS81 in human cells, EME1 and EME2 (**Figure 2C**; Ciccia et al., 2003). While both complexes can process branched DNA structures *in vitro*, MUS81-EME2 exhibits higher nuclease activity and broader substrate specificity (Ciccia et al., 2007; Amangyeld et al., 2014; Pepe and West, 2014b). MUS81 and EME1 display increased levels of phosphorylation in prometaphase nocodazole-arrested cells compared to asynchronous, thymidine-(G1/S) or CPT-arrested (S/G2) cells. Given the coincidence of such modifications and an increase in the catalytic activity of MUS81 immunoprecipitates, it was put forward that similar regulatory mechanisms might operate to control MUS81 function in *S. cerevisiae* and in humans (Matos et al., 2011).

In terms of protein–protein interactions, MUS81 is also known to directly associate with SLX4. Together with SLX1, SLX4 constitutes a SSE with the ability to process recombination intermediates *in vitro* and *in vivo* and serves as a landing platform for other DNA repair factors like XPF-ERCC4 (Andersen et al., 2009; Fekairi et al., 2009; Munoz et al., 2009; Svendsen et al., 2009; Wechsler et al., 2011). The MUS81-SLX4 interaction is mediated through residues within the N-terminal region (1–86) of MUS81 and the SAP domain (a putative DNA-binding region) of SLX4 and plays an important role in both general HR repair as well as in CPT- and PARP inhibition-induced DNA damage repair (Fekairi et al., 2009; Castor et al., 2013; Garner et al., 2013; Kim et al., 2013). Mutations such as W24A/L25A and L66A/Q67A in the murine ortholog of MUS81 can disrupt the interaction with SLX4 without affecting the stability of the MUS81-EME1 complex or its nucleolytic activity on 3'-flaps (Nair et al., 2014). Likewise, the Y1340A, L1348A, and E1351A/L1352A mutations in the SLX4 SAP domain from mice could specifically abolish the MUS81-SLX4 interaction without disrupting the SLX4 ability to coimmunoprecipitate SLX1 and ERCC1 (Castor et al., 2013).

Interestingly, the increase in HJ-processing activity observed in MUS81 immunoprecipitates from cells arrested with nocodazole is dependent on CDK activity and requires SLX4 (Wyatt et al., 2013). SLX4, like MUS81 and EME1, is phosphorylated in a CDK1-dependent manner and inhibition of CDK kinase activity in nocodazole-arrested cells triggers dissociation of the complex. Therefore, it has been suggested that the increased capability of HJ resolution in mitotic MUS81 immunoprecipitates may arise from the coordination of different nucleases on the SLX4 scaffold (Wyatt et al., 2013), although the molecular basis for the CDK1-driven interaction of these two proteins remains unclear. In this sense, biochemical experiments have shown that full-length recombinant SLX1-SLX4 and MUS81-EME1 complexes can interact with each other *in vitro* to form a more efficient HJ resolvase. Quantitatively, the complex of the two nucleases displays higher HJ-processing activity than the sum of both nucleases separately, with a particular stimulation of the initial rate of the reaction. Qualitatively, the SLX1-SLX4-MUS81-EME1 complex carries out a more coordinated HJ resolution reaction, as judged by the increased rate of bilateral cleavage and linear product formation in a plasmid-borne cruciform cleavage assay (Wyatt et al., 2013). These results indicate that recruitment of the MUS81 nuclease to the SLX4 scaffold can improve its HJ resolution activity by coordinating its actions with those of SLX1.

Finally, another layer of complexity in the regulation of the MUS81 nuclease arises from the existence of the non-catalytic subunit EME2. While EME1 associates with MUS81 throughout the cell cycle (Matos et al., 2011; Wyatt et al., 2013), the MUS81-EME2 complex is detectable predominantly during S-phase (Pepe and West, 2014a). Therefore, the usage of alternative non-catalytic subunits may play a significant role in the regulation of MUS81, with MUS81-EME2 being involved in earlier events like replication fork restart, but not in later roles like the removal of HJs (Pepe and West, 2014a). We anticipate that forthcoming studies will refine our knowledge about the MUS81 partner choice (EME1 vs. EME2) and its connection to the distinct cellular functions of the two MUS81 complexes.

The Yen1/GEN1 Nucleases

Human GEN1 and *S. cerevisiae* Yen1 are ortholog enzymes that belong to the subclass IV of the XPG/Rad2 family of SSEs (Johnson et al., 1998; Furukawa et al., 2003; Ip et al., 2008). Like all the other members of this family, they contain a bipartite nuclease domain, constituted by the XPG-N and XPG-I subdomains connected by a poorly conserved linker region (Lieber, 1997). While this is the main area for the interaction between the protein and the branched DNA region, another conserved feature of the family, the helix-two-turn-helix motif, stabilizes DNA binding through its contacts with the duplex DNA portion of the substrates (Tsutakawa et al., 2011, 2014). Yen1 and GEN1 were characterized as the first eukaryotic nucleases that processed HJs in a similar manner to the archetypical bacterial RuvC HJ resolvase (Ip et al., 2008). A recent report has shown that the two members of this subclass IV in *A. thaliana*, AtGEN1 and AtSEND1, also possess HJ resolution activity (Bauknecht and Kobbe, 2014), supporting the hypothesis that this subclass IV of the XPG/Rad2 family comprises a group of enzymes that have evolved HJ resolution activity (Ip et al., 2008). Interestingly, all these HJ resolvases retain the characteristic 5'-flap processing activity of the family, while they can also target other replication fork-like structures (Kanai et al., 2007; Ip et al., 2008; Rass et al., 2010; Yang et al., 2012; Bauknecht and Kobbe, 2014; Freeman et al., 2014). Therefore, as with MUS81, such potential for the cleavage of fully double-stranded replication or recombination intermediates could explain why cells have implemented mechanisms to tame these inherently dangerous activities.

S. cerevisiae Yen1

Two distinct layers of cell-cycle stage-specific regulation govern Yen1 function: subcellular localization and biochemical activation. The basis for this regulation relies on changes in the phosphorylation status of the protein, which are imposed by two master regulators of the cell cycle: Cdc28/Cdk kinase and Cdc14 phosphatase (Blanco et al., 2014; Eissler et al., 2014; Garcia-Luis et al., 2014). At the onset of S-phase, phosphorylation of Yen1 drives its exclusion from the nucleus, at the same time that it inhibits its nuclease activity. When cells enter anaphase, Cdc14 is released from the nucleolus and dephosphorylates Yen1, which re-enters the nucleus and becomes catalytically active (Kosugi et al., 2009; Matos et al., 2011, 2013; Blanco et al., 2014; Eissler et al., 2014; Garcia-Luis et al., 2014).

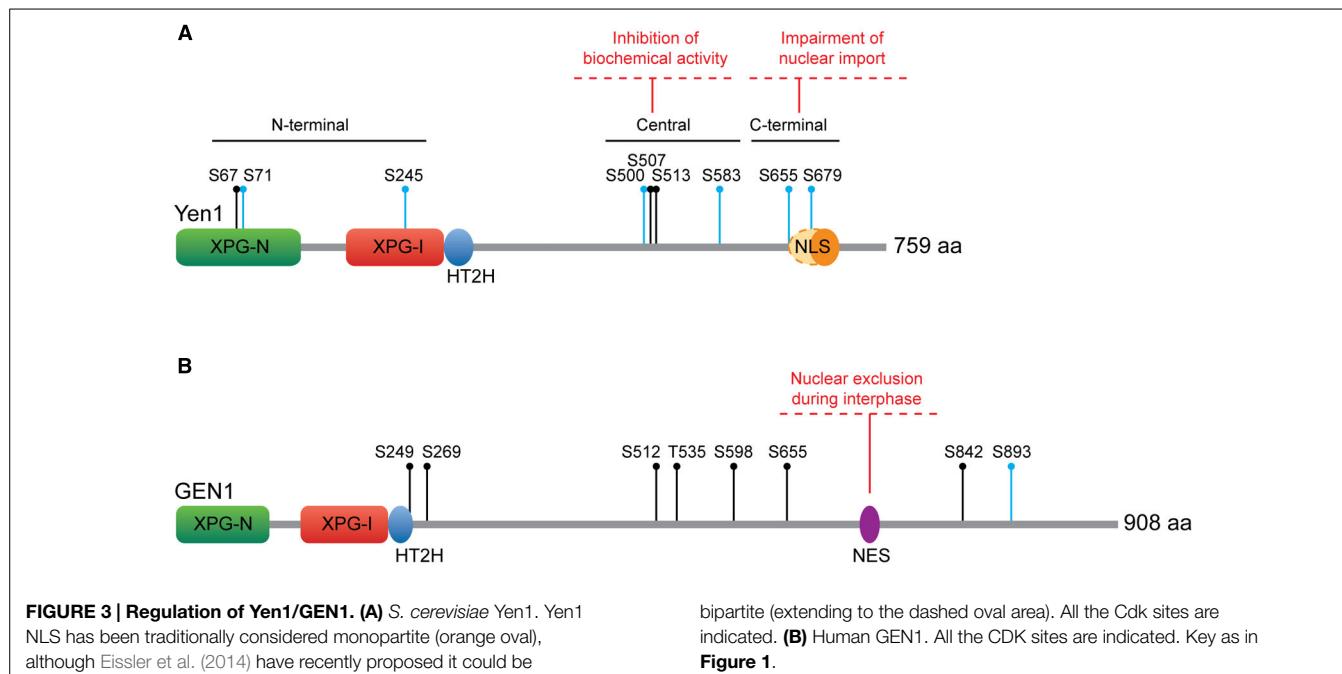
Yen1 contains nine consensus Cdk sites (S-P, all serines), with eight of them being full Cdk sites S-P-X-K/R. These Cdk sites show some degree of clustering in each of the N-terminal, central and C-terminal regions of the protein (Figure 3A), a predictive feature of bona fide Cdk substrates (Moses et al., 2007). Indeed, Yen1 was confirmed as a target for Cdk-dependent phosphorylation in whole-cell extracts through proteome-wide approaches (Ubersax et al., 2003), being a particularly good substrate for the S-phase complex Cdc28-Clb5 (Loog and Morgan, 2005). Six out of these nine Cdk sites have been verified as phosphoresidues *in vivo* by mass spectrometry (Blanco et al., 2014; Eissler et al., 2014). Additionally, four of the Cdk sites were identified as optimal targets for Cdc14 (S500, S507, S655, and S679) through *in silico* prediction and *in vitro* analyses of

Cdc14 activity on peptides containing these phosphoresidues (Figure 3A; Eissler et al., 2014).

With regard to the control of Yen1 nuclease activity, it was initially observed that the ability of Yen1 to process a HJ substrate fluctuates throughout the cell cycle. While immunoprecipitates of Yen1 from cells in S-phase show low levels of nuclease activity, those from cells in mitosis can efficiently process synthetic HJs (Matos et al., 2011). Mutation of the nine CDK consensus sites in Yen1 to alanine results in a protein [Yen1-9A (Eissler et al., 2014) or Yen1^{ON} (Blanco et al., 2014)] that, as opposed to the wild-type, (i) cannot be phosphorylated by Cdk, (ii) does not interact with Cdc14 and (iii) displays a maximum level of activity during all phases of the cell cycle, bypassing the requirement for Cdc14 for its activation. A partial dissection of the relative contribution of the different Cdk sites to this regulation has shown that serine to alanine mutations in the C-terminal cluster (S655 and S679) have no effect on the biochemical activity of Yen1 and, so far, the significance of the potential phosphorylation events on the N-terminal cluster remains poorly defined. However, phosphorylation-resistant mutants in the four serines of the central cluster (S500, S507, S513, and S583) result in a protein that is no longer inhibited during S-phase and displays increased levels of crossover formation (Blanco et al., 2014; Eissler et al., 2014). Conversely, expression of a phosphomimetic mutant for these residues phenocopies the deletion of *YEN1* (Eissler et al., 2014). Since phosphorylation-dependent inhibition of Yen1 nuclease activity derives from a reduction in its substrate binding affinity (Blanco et al., 2014), the central cluster may be part of a repressible DNA binding domain or serve as a switch for a conformational change between low- and high-DNA binding forms of Yen1.

Concerning the spatial regulation of Yen1, the changes in its localization are related to the phosphorylation status of the CDK target site S679. This serine in the C-terminal cluster overlaps with a predicted nuclear localization signal (NLS; 679-SPIKKSRTT-687). Immunofluorescence analysis of overexpressed, GFP-tagged Yen1 revealed that the mutation of S679 to alanine led to permanent nuclear accumulation (Kosugi et al., 2009). A more detailed analysis showed that phosphorylation of other CDK sites may also influence Yen1 localization. The proportion of nuclear Yen1 was shown to be higher with Yen1^{ON} than with Yen1^{S679A} mutants (Blanco et al., 2014). Therefore, it has been suggested that the NLS in Yen1 may be bipartite and controlled by the phosphorylation of two different CDK sites, S655, and S679 (Eissler et al., 2014). In addition, the observation that Msn5, a karyopherin involved in the nuclear export of phosphorylated proteins, is responsible for Yen1 export (Kosugi et al., 2009), suggests that the phosphorylation status of CDK sites overlapping yet-unidentified nuclear export signals may also influence its localization.

The expression of Yen1^{ON}/Yen1-9A, which bypasses the two levels of Cdk-dependent control, has demonstrated the importance of restricting Yen1 function prior to anaphase for the maintenance of genome stability. Premature activation of Yen1 leads to increased DNA-damage sensitivity, crossover formation and loss of heterozygosity in diploid cells (Blanco et al., 2014; Eissler et al., 2014). Incidentally, Yen1^{ON} can



suppress the synthetic lethality of *mus81Δ sgs1Δ* double mutants, thus demonstrating that the premature activation of Yen1 can compensate for the loss of other genes involved in the processing of recombination intermediates (Blanco et al., 2014).

Human GEN1

Soon after its identification, several lines of evidence pointed toward proteolytic cleavage as a putative mechanism to regulate GEN1 activity through the excision of a self-inhibitory domain in the long, unstructured C-terminal region of the protein (Ip et al., 2008; Rass et al., 2010). Both the ~60 kD N-terminal fragment originally identified by mass spectrometry of highly fractionated HeLa extracts and the recombinant GEN1^{1–527} truncation fragment exhibited increased biochemical activity compared to the full-length protein (Ip et al., 2008). Moreover, GEN1^{1–527} was able to partially suppress the DNA damage sensitivity and meiotic crossover defects of fission yeast strains deficient for either Mus81 or Rqh1 (Sgs1 in budding yeast, BLM in mammals; Lorenz et al., 2010). However, there is no definite evidence so far for the C-terminal region cleavage as an activation mechanism for GEN1 *in vivo*.

On the other hand, the sequence of GEN1 contains eight CDK consensus target sites, a number similar to those in Yen1, although their relative position and context is not conserved (Figure 3B). This suggested that a similar CDK phosphorylation-dependent regulatory mechanism could operate for Yen1 and GEN1 (Matos et al., 2011). It has been recently shown that GEN1 is indeed phosphorylated in a seemingly CDK-dependent manner, as mutation of all the serines/threonines in its consensus CDK targets (GEN1^{8A}) abolishes virtually all the slowly migrating forms of the protein (Chan and West, 2014). However, changes in the phosphorylation status of the protein do not affect its nuclease activity as dramatically as in the case of Yen1, since

both GEN1^{8A} and *in vitro* dephosphorylated GEN1 retain the wild-type ability to process HJs (Matos et al., 2011; Chan and West, 2014). Therefore, it appears unlikely that the control of the biological functions of GEN1 relies on the direct modulation of its nucleolytic activity. Instead, localization studies have suggested that human cells restrict the actions of this nuclease through its temporal exclusion from the nucleus. GEN1 is strongly enriched in the cytoplasm during interphase, gaining access to chromatin in prometaphase, only after the nuclear envelope has broken down (Matos et al., 2011). The subcellular localization pattern of GEN1 appears to be exclusively dependent on a nuclear export signal that has been recently identified in its unstructured C-terminal region (660-LLSGITDLCL-669; Chan and West, 2014).

To demonstrate the importance of the restriction of GEN1 access to the nucleus prior to mitosis, a constitutively nuclear version of the enzyme, GEN1^{nuc}, was generated by adding three copies of a SV40-derived NLS at its C-terminus and by mutating four key hydrophobic residues in GEN1 NES to alanine (L660A, L661A, I664A, L667A). When introduced in cells, GEN1^{nuc} induces a series of phenotypes that are partially reminiscent of those observed in yeast expressing mis-regulated Yen1. For instance, GEN1^{nuc} expression increases the occurrence of sister chromatid exchanges (COs). Also, it can reduce the defects associated with the double depletion of MUS81 and BLM, resulting in increased cellular viability and reduction of chromosomal breaks (Chan and West, 2014). However, no increased sensitivity to DNA-damaging agents was observed in cells expressing GEN1^{nuc}. This could reflect a differential ability between Yen1 and GEN1 to process branched DNA structures that are generated *in vivo* during active replication, stalled replication fork repair/restart or the early steps of homologous recombination (Blanco et al., 2014; Chan and West, 2014).

Concluding Remarks

Traditional models of homologous recombination based on a DNA substrate-centric view of JM-processing (e.g., **Figure 1**) are insufficient to explain how cells control the outcome of DNA repair. In such models, enzymes are positioned according to their *in vitro* substrate preference, which is difficult to reconcile with their rather promiscuous biochemical properties and intricate genetic relationships. Work on the regulation of MUS81* and Yen1/GEN1 nucleases has introduced a new dimension to such models, a dimension in which defined cellular circumstances strongly influence pathway usage. However, despite the recent advances summarized here, there are still fundamental questions concerning SSE regulation that remain unanswered. For instance, we have just begun to comprehend the mechanistic basis for the control of the catalytic properties of SSEs by post-translational modifications. Therefore, we will need detailed biochemical and structural information to help us understand how similar phosphorylation events translate into opposite responses from each protein.

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The ability to turn on and off JM-processing enzymes at a given cell cycle stage, or upon the cellular detection of a given stimulus, seems an efficient mechanism to bias the choice toward the most suitable DNA repair pathway and could potentially control the function of repair enzymes other than MUS81* and Yen1/GEN1. It is therefore tempting to envisage that in addition to developing enzymes capable of processing a specialized, but overlapping range of DNA substrates, cells have evolved the general ability to regulate their actions. Such combination of capacities would prevent pathway competition as well as the toxic processing of vital endogenous DNA structures. Furthermore, it would allow for the flexible implementation of pathway usage according to the chromosome segregation programs of meiosis and mitosis.

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Is homologous recombination really an error-free process?

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Homologous recombination (HR) is an evolutionarily conserved process that plays a pivotal role in the equilibrium between genetic stability and diversity. HR is commonly considered to be error-free, but several studies have shown that HR can be error-prone. Here, we discuss the actual accuracy of HR. First, we present the product of genetic exchanges (gene conversion, GC, and crossing over, CO) and the mechanisms of HR during double strand break repair and replication restart. We discuss the intrinsic capacities of HR to generate genome rearrangements by GC or CO, either during DSB repair or replication restart. During this process, abortive HR intermediates generate genetic instability and cell toxicity. In addition to genome rearrangements, HR also primes error-prone DNA synthesis and favors mutagenesis on single stranded DNA, a key DNA intermediate during the HR process. The fact that cells have developed several mechanisms protecting against HR excess emphasize its potential risks. Consistent with this duality, several pro-oncogenic situations have been consistently associated with either decreased or increased HR levels. Nevertheless, this versatility also has advantages that we outline here. We conclude that HR is a double-edged sword, which on one hand controls the equilibrium between genome stability and diversity but, on the other hand, can jeopardize the maintenance of genomic integrity. Therefore, whether non-homologous end joining (which, in contrast with HR, is not intrinsically mutagenic) or HR is the more mutagenic process is a question that should be re-evaluated. Both processes can be “Dr. Jekyll” in maintaining genome stability/variability and “Mr. Hyde” in jeopardizing genome integrity.

Keywords: Homologous recombination, mutagenesis, DNA double strand break repair, replication stress, genetic variability, genetic instability

INTRODUCTION

Genomes are routinely challenged with exogenous or endogenous insults of enzymatic, chemical or physical origins. These DNA alterations can generate genetic instability, leading to cell death, senescence, developmental abnormalities and tumor initiation and progression. However, while it is vital to maintain genomic stability, genetic diversity is essential to physiological processes, such as the generation of the immune repertoire or the mixing of parental alleles during meiosis. Additionally, the absence of genetic diversity would constitute an evolutionary dead end. Thus, DNA repair should maintain genomic stability and allow for genetic diversity. Therefore, the accuracy of DNA repair processes is an essential issue.

Homologous recombination (HR) is a process that is conserved in all organisms, playing an essential and pivotal role in genome stability and plasticity. Notably, HR is involved in the reactivation of replication forks that have been blocked and in the repair of DNA double strand breaks (DSBs) (reviewed in Haber, 2014).

Replication fork progression is routinely challenged by diverse exogenous or endogenous stresses, which ultimately leads to replication fork stalling, collapse or breakage, and triggers the DNA damage response (DDR) (Hyrien, 2000; Lambert and Carr, 2005, 2013; Tourriere and Pasero, 2007; Aguilera and Garcia-Muse,

2013). Failures in chromosome replication are thus a primary source of genetic instability. Consistently, in many organisms, including yeast and human cells, both slowing down and blocking fork progression are associated with chromosome breakage and genome rearrangement (reviewed in Aguilera and Gomez-Gonzalez, 2008; Branzei and Foiani, 2010). Moreover, impediments to fork progression might also challenge the completion of DNA replication, resulting in mitotic defects and multipolar mitotic cells, which then lead to uneven chromosome segregation and thus amplifying the genome instability to the whole genome, including fully replicated regions (Wilhelm et al., 2014). Consistently with the existence of endogenous replication stresses, DDR activation resulting from spontaneous endogenous replication stress has also been detected in the early stages of carcinogenesis and senescence (Bartkova et al., 2005, 2006; Gorgoulis et al., 2005; Halazonetis et al., 2008; Gorgoulis and Halazonetis, 2010).

DSBs are harmful lesions that are produced through exposure to exogenous treatments, such as ionizing radiation (IR), byproducts of endogenous cellular metabolism and, importantly, replication forks arrest (Seigneur et al., 1998; Featherstone and Jackson, 1999; Saintigny et al., 2001; Rothkamm and Lobrich, 2003; Mahaney et al., 2009). DSBs can trigger profound genomic rearrangements or, in contrast, generate genetic diversity in

essential biological processes. In the latter case, programmed DSBs are physiologically produced through controlled cellular enzymes during meiotic differentiation, mating-type switching in *Saccharomyces cerevisiae* or in V(D)J and class switch recombination, which ensures the diversity of the immune response (reviewed in Haber, 1992; Jung and Alt, 2004; Lieber et al., 2004; Rooney et al., 2004; Dudley et al., 2005; Buard and de Massy, 2007).

Two primary strategies are used to repair DSBs: (1) HR, which requires a sequence-homologous partner and, in fact, corresponds to different processes involving both common and distinct mechanisms (see below and **Figure 1**); and (2) NHEJ (non-homologous end joining), which ligates the DNA ends of a DSB without requiring extended homologies (Haber, 2014). Note that a highly mutagenic alternative end-joining pathway (A-EJ) has recently been identified (for review Grabarz et al., 2012; Rass et al., 2012; Betermier et al., 2014).

In most of the literature, HR is described as an error-free process, while NHEJ is described as an error-prone DSB repair process. This statement is largely based on the fact that the mechanism of HR requires the search for a homologous partner to repair DNA, in contrast to NHEJ. Careful examination of the data from the literature might challenge these assumptions, which requires revisiting the current view. Indeed, recent data points to the intrinsic precision of canonical NHEJ (C-NHEJ; KU-Ligase 4-dependent) in contrast to A-EJ. In fact, C-NHEJ is conservative but adaptable, and the accuracy of the repair is dictated by the structure of the DNA ends rather than by the C-NHEJ machinery itself (Grabarz et al., 2012; Rass et al., 2012; Betermier et al., 2014).

Here, in a reciprocal view, we discuss the accuracy of HR and we present several situations of mutagenesis generated by HR. We conclude that HR is a double-edged sword, which on the one hand controls the equilibrium of genomic stability vs. diversity, but on the other hand can jeopardize the maintenance of genomic integrity. The importance of the versatility of HR and its impact on genomic integrity are discussed.

THE PRODUCTS OF HR (GENE CONVERSION AND CROSSING OVER) AND MODELS

Consistently with the implication of HR in genome stability maintenance, mutant cells that are defective in HR show elevated mutagenesis and genetic instability. However, in contrast, HR can appear as a mutagenic process *per se*, in many situations. Such concepts can be understood when considering the products and molecular mechanisms of HR.

The products of HR are gene conversion (GC: non-reciprocal exchange of genetic material) associated or not with crossing-over (CO: reciprocal exchange of the adjacent sequences) (**Figure 1A**). Such products can account for genetic diversity or instability arising through HR.

MODELS OF HR FOR DSB REPAIR

All HR processes are initiated through the 5' to 3'-single-stranded resection of double stranded DNA ends, creating a 3'-single-stranded DNA (ssDNA), on which the pivotal RecA/Rad51 recombinase is loaded (**Figure 1B**). The RecA/Rad51 nucleofilament carries out the subsequent invasion

of a homologous DNA duplex that primes DNA synthesis and copies the intact DNA molecule. At this point, the HR processes differ in the processing of the intermediates, leading to either gene conversion, associated or not with crossing-over, or to SDSA (synthesis-dependent strand annealing) and BIR (break-induced replication) (**Figure 1B**). In addition, an alternative process (SSA, single-strand annealing) is also initiated by resection; however, the following step does not require Rad51 nor strand invasion of an intact duplex DNA, but the annealing of two complementary ssDNAs (**Figure 1C**). SSA is a non-conservative process that systematically leads to the deletion of the intervening sequence between the two interacting DNA molecules (reviewed in Haber, 2014).

HR AND REPLICATION FORKS REACTIVATION

HR contributes to the robustness of DNA replication by multiple mechanisms (**Figure 2**) and might be viewed as a pathway escorting fork progression (reviewed in Costes and Lambert, 2012) (**Figure 2**). HR can act either at replication forks or at replicated chromatids to ensure the completion of chromosome duplication. First, HR efficiently seals ssDNA gaps that have been left within replicated chromatids after fork passage through DNA lesions. Second, HR is involved in the recovery of arrested replication forks and has the potential to reassemble a functional replisome. While the mechanism of origin-independent loading of a replisome by HR has been extensively characterized in bacteria, its counterpart in eukaryotic cells has only recently begun to emerge.

Fork passage over a ssDNA nick or gaps in the parental DNA leads to a broken fork, with one of the sister chromatids being disconnected from the fork. Some components of the replisome are thus lost (Roseaulin et al., 2008; Hashimoto et al., 2010; Moriel-Carretero and Aguilera, 2010). HR ensures the repair of such broken forks through a mechanism that is thought to be similar to BIR (Bosco and Haber, 1998; Kraus et al., 2001; Hashimoto et al., 2010). In *Xenopus*, HR-mediated fork repair leads to the reassembly of a replisome (Hashimoto et al., 2012). But BIR that requires most of the components of canonic replisomes (Lydeard et al., 2007, 2010) is highly mutagenic in yeast (Deem et al., 2011). An inter-strand cross-link (ICLs) is a type of lesion that interferes with the progression of replication forks by preventing the unwinding of the parental DNA. ICLs are cleaved by specific nucleases, thus resulting in a broken fork that is then repaired by HR (Long et al., 2011).

Many chromosomal elements can behave as fork obstacles, and it remains unclear whether fork breakages occur systematically. For example, DNA-bound proteins represent more than 1400 potential sites of fork arrest in budding yeast, and HR efficiently rescues replication forks blocked by protein complexes tightly bound to DNA in fission yeast (Ivessa et al., 2003; Lambert et al., 2010; Iraqui et al., 2012). In this case, replication restart is initiated by the loading of HR factors at ssDNA exposed at blocked forks (Mizuno et al., 2009; Lambert et al., 2010). The mechanisms by which HR ensures replication restart remain to be determined. Nevertheless, the resumption of DNA synthesis at inactivated forks via the HR pathway is also mutagenic (see below).

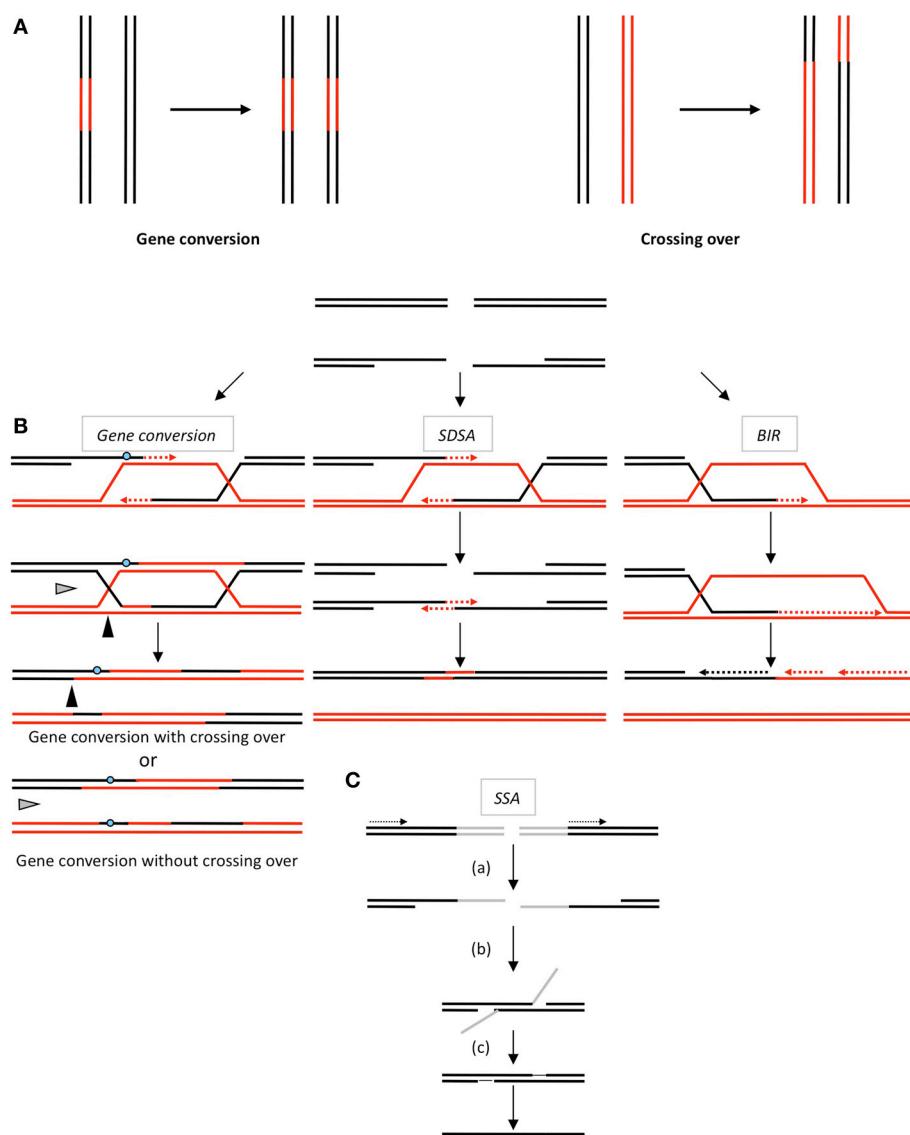
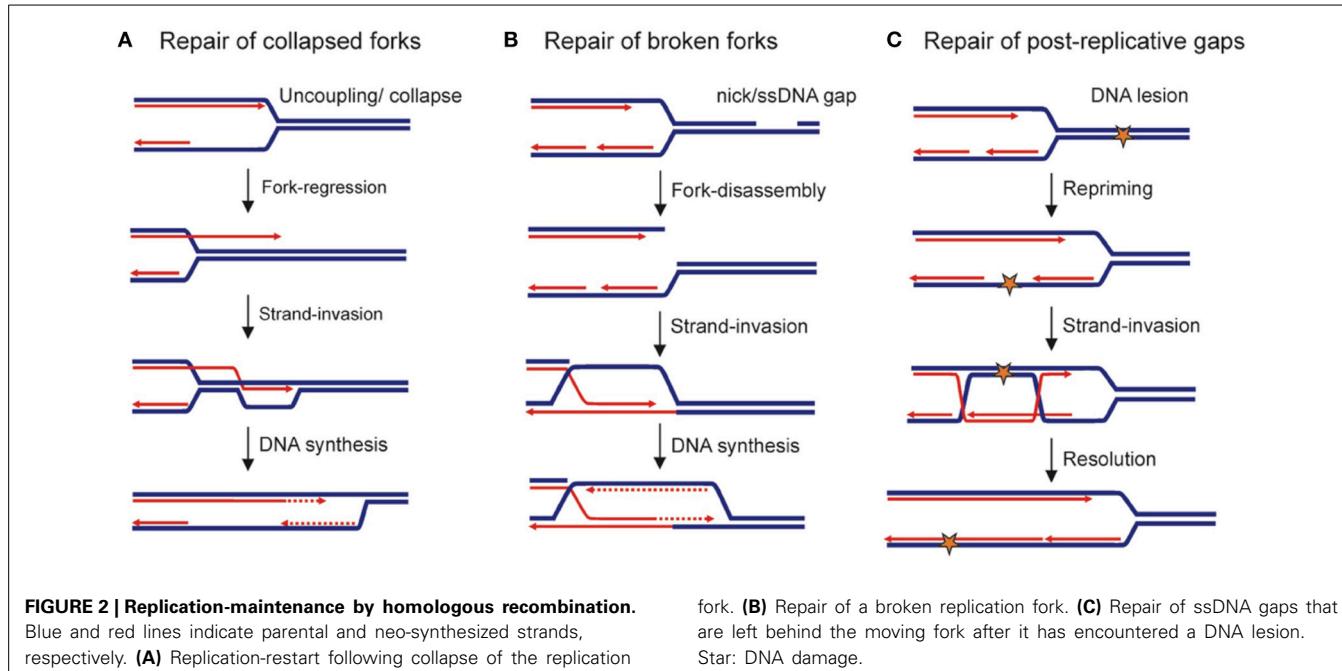


FIGURE 1 | (A) The products of HR. Gene conversion (left panel) leading to non-reciprocal exchange of a DNA sequence (in red). Crossing over (right panel): reciprocal exchanges of adjacent sequences (black and red). Note that gene conversion can be associated with or without crossing over. **(B)** The double-strand break repair models through HR. Left panel: Gene conversion. After resection, the single-stranded 3'-tail invades a homologous, intact double-stranded DNA, forming a D-loop (displacement loop). This process tolerates limited imperfect sequence homologies, thus creating heteroduplex intermediates bearing mismatches (blue circle). The invading 3'-end primes DNA synthesis, which then fills in the gaps. The cruciform junctions (Holliday junctions, HJ) migrate. Resolution (or dissolution) of the HJ occurs in two different orientations (black or gray triangles), resulting in gene conversion either with or without crossing

over. Middle panel: Synthesis-dependent strand annealing. Initiation is similar to that of the previous model, but the invading strand de-hybridizes and re-anneals at the other end of the injured molecule; no HJ is formed. Right panel: Break-induced replication (BIR). The initiation is similar to that of the previous models, but the synthesis continues over longer distances on the chromosome arms, even reaching the end of the chromosome. Here, there is neither resolution of the HR nor crossover. **(C)** Single-strand annealing (SSA). When a double-strand break is generated between two homologous sequences in tandem in the same orientation (dotted arrows), an extended single-strand resection (a) reveals two complementary DNA strands that can hybridize (b). (c) Resolution of the intermediate and gap filling complete the repair, leading to the deletion of the intergenic sequences between the initial repetitions.

Finally, in addition to rescuing DNA synthesis at replication forks, HR is also involved in the stability and protection of forks that are impeded in their progression. HR defects lead to the accumulation of ssDNA gaps at replication forks, perhaps due to an uncoupling between lagging and leading strand synthesis (Hashimoto et al., 2010). Additionally, resection

of neo-synthesized strands has been observed in mammalian and bacterial HR-deficient cells (Courcelle and Hanawalt, 2003; Schlacher et al., 2011). While this fork-stabilizer function of HR during DNA replication appears to be evolutionarily conserved, its importance in ensuring the robustness of DNA replication remains to be established in eukaryotes.



Therefore, because HR acts through multiple pathways at the replication fork or in its vicinity, it should play an essential role in protecting cells against spontaneous replication stress and thus against the resulting genetic instability, as discussed below.

ROLE OF HR IN THE MAINTENANCE OF GENOME STABILITY

HR DEFECTS RESULT IN HIGHER LEVELS OF MUTAGENESIS AND GENETIC INSTABILITY

In all organisms, HR-deficient cells exhibit a higher level of mutagenesis and genome rearrangements, both spontaneous and upon exposure to exogenous genotoxic agents (Quah et al., 1980; Liu et al., 1998; Takata et al., 2001; Thompson and Schild, 2001; Lambert and Lopez, 2002; Popova et al., 2012). These data suggest that HR (like NHEJ) maintains genome stability.

HR PROTECTS MITOSIS FROM REPLICATION STRESS

Replication stress covers many events that impact the accuracy of DNA replication and then jeopardize chromosome segregation during mitosis. Low levels of replication stress can generate mitotic defects, including anaphase bridges, supernumerary centrosomes and multipolar mitosis, which then lead to uneven chromosome segregation (Wilhelm et al., 2014). Because HR plays a pivotal role in the resumption of arrested replication forks, defects in HR should thus reveal endogenous replication stress. Consistently, HR-deficient cells are associated with spontaneous slowed replication fork progression (Daboussi et al., 2008; Wilhelm et al., 2014), anaphase bridges (Lahkim Bennani-Belhaj et al., 2010; Laulier et al., 2011b; Rodrigue et al., 2013; Wilhelm et al., 2014), common fragile sites (Ingvarsson et al., 1999; Turner et al., 2002), supernumerary centrosomes (Griffin et al., 2000; Deng, 2002; Kraakman-van der Zwet et al., 2002; Bertrand et al., 2003; Dodson et al., 2004; Daboussi et al., 2005; Katsura et al., 2009; Plo and Lopez, 2009; Rodrigue et al., 2013; Wilhelm et al., 2014), and multipolar mitosis (Wilhelm et al.,

2014). Similarly, fission yeast recombination factors are necessary to ensure successful chromosome segregation following the slowdown of fork progression (Bailis et al., 2008).

These data underline the essential role played by HR in protecting genome stability at the interface between replication and mitosis, as reviewed elsewhere (Wilhelm et al., 2014).

HR: A FACTOR OF GENETIC INSTABILITY

Because of its intrinsic properties (genetic exchanges through GC and CO), HR can generate genetic instability. More surprisingly, several reports have noted a type of genome instability mediated by micro-homology in an HR-dependent manner. These types of genetic instability were initially assigned to the error-proneness of end joining. Consequently, the actual view on the accuracy of HR has been challenged in many reports.

HR POSSESSES THE INTRINSIC CAPACITY OF GENETIC MODIFICATION

HR is initiated through the invasion of a duplex DNA by a homologous single-stranded molecule, which then primes DNA synthesis (Figure 1B). The strand invasion, promoted by RecA/Rad51, is able to occur with homologous sequences containing few heterologies (although the divergences should be limited), thus generating heteroduplex DNA molecules bearing mismatches (Figure 1B). The repair of these mismatched structures can transfer sequence polymorphisms and modify the genetic information of the recipient molecule, resulting in an apparent mutagenic event. Additionally, the DNA synthesis initiated by the invading strand (Figure 3A) can duplicate a sequence that was absent in the donor molecule and thereby transfer this genetic information, resulting in modifications of the original recipient DNA sequence. Moreover, the resolution of the HR intermediate (Holliday junctions) can facilitate the exchange of adjacent sequences, leading to genetic rearrangements. Thus, both GC and CO intrinsically possess the capacity to modify genetic information. This has been

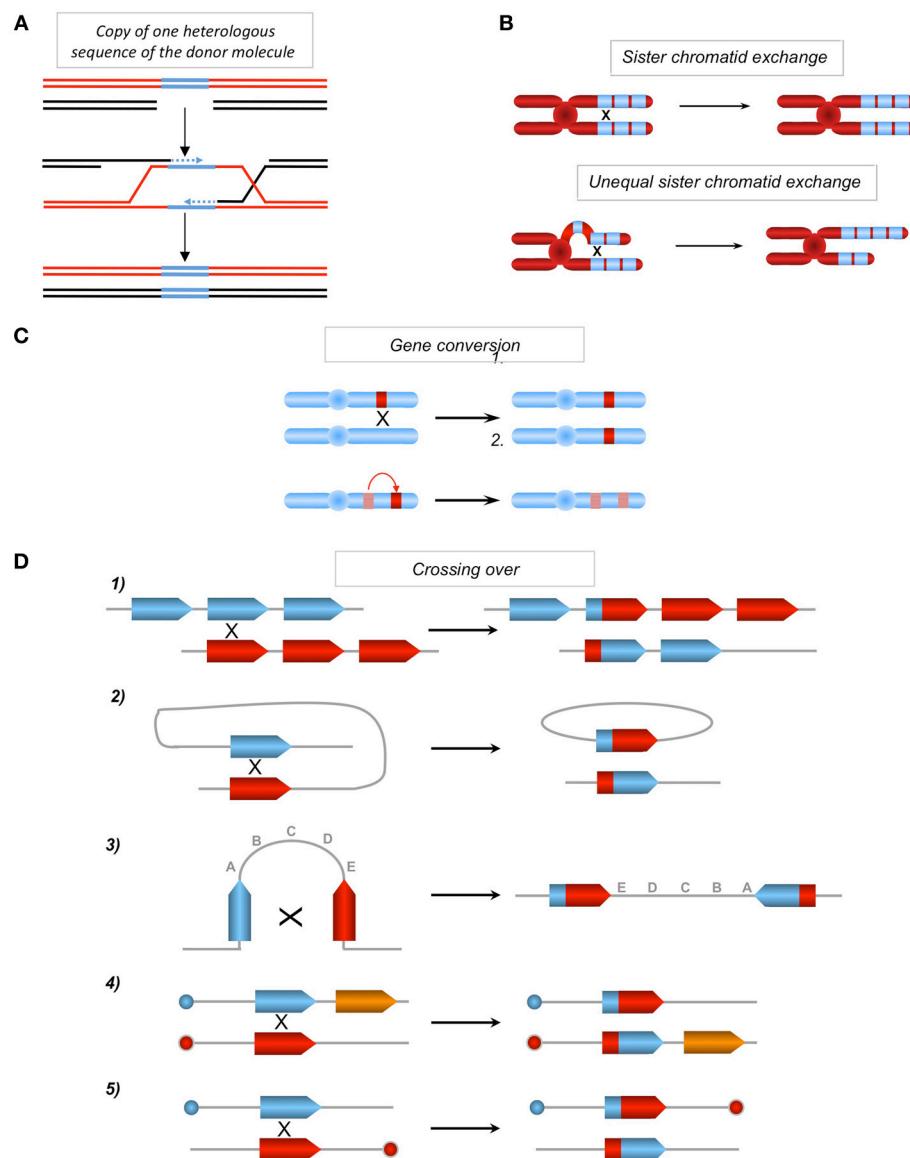


FIGURE 3 | (A) Copy of one sequence of the donor absent on the recipient molecule. One of two homologous molecules (red and black) can contain one heterologous sequence (blue). Upon gene conversion or SDSA (see Figure 1) the heterologous (blue) sequence can be copied and transferred from the donor sequence (red) to the homologous recipient sequence (black), resulting in a genetic modification of the recipient sequence. **(B)** Sister chromatid exchanges. Between repeat sequences (blue boxes) without misalignment (upper panel) or with misalignment resulting in unequal sister chromatid exchanges (lower panel) and amplification and loss of genetic material. **(C)** Impact of gene conversion. Non-reciprocal exchange of genetic information between two heteroalleles, leading to a loss of heterozygosity (upper panel) and between a pseudogene (hatched), which often contains nonsense

mutations and a gene (in red), leading to the inactivation of the latter (lower panel). **(D)** Chromosomal rearrangements resulting from crossing-over (CO) between repeat sequences. (1) Between homologous sequences on two chromosomes or following unequal sister chromatid exchange on the same chromosome, resulting in the amplification of one molecule and the deletion of the other. (2) Intramolecular CO between two homologous sequences in a direct orientation, resulting in the excision of the intervening sequence. (3) Intramolecular CO between two homologous sequences in an inverted orientation, resulting in the inversion of the internal fragment. (4) and (5) Inter-chromosomal CO, depending upon the orientation of the homologous sequences with respect to their centromeres (blue or red circles); this process generates a translocation (4) or a dicentric and an acentric chromosome (5).

used to target gene replacement and gene correction using exogenous DNA. Note that when involving identical sequences (for instance sister chromatids exchange: SCE), HR does not impact the genetic information. However, unequal SCE can lead to sequence duplication or deletion (Figure 3B). One can object that

unequal SCEs should be less frequent than equal SCEs (Gonzalez-Barrera et al., 2003). Therefore, genome stability should not be strongly impacted by SCEs. In contrast, when involving repeated sequences (which are not identical) dispersed throughout the genome (non-allelic recombination, NAHR), HR can affect the

genetic information (see below). Note that, if the final product of an equal SCE is error-free, this is not due to the accuracy of the HR process, but to the fact that the DNA are identical (indeed HR can efficiently processes with imperfectly homologous sequences) and because associated mechanisms orientate such kinds of events: 1-HR is restricted to the S and G2 phases (which correspond to the cell cycle phases presenting sister chromatids) and 2-the tight cohesion of the sister chromatids, through the cohesins complex, orientates the event to an equal SCE. Thus, the structure of the DNA and accessory associated mechanisms, rather than HR itself, favor such an error-free event. In addition, HR can initiate mutagenic DNA synthesis even when the interacting DNA molecules are fully identical such as sister chromatids (see discussion below). Finally, we can point out that, in yeast as well as in mammalian cells, spontaneous SCE have been described to be largely independent of the main actors of HR (Rad51, Rad52, Rad54), in contrast with induced SCE (Dronkert et al., 2000; Fasullo et al., 2001; Lambert and Lopez, 2001; Dong and Fasullo, 2003). Noteworthy, at meiosis, which aims at creating genetic diversity, equal SCEs are repressed and HR between homologous chromosomes (which are not identical) is favored. Therefore, in this situation, HR is used to generate genetic diversity.

Thus, in the cases discussed above, associated processes, rather than the HR machinery itself, in fact control the accuracy of the final outcome of HR.

GENETIC ALTERATIONS THROUGH GC AND/OR CO

Gene conversion is able to transfer genetic information in a non-reciprocal manner between two hetero-alleles, resulting in loss of heterozygosity; gene conversion can also transfer one stop codon from a pseudogene to a related coding sequence, leading to its extinction (**Figure 3C**) (Amor et al., 1988; Fusco et al., 2012). Moreover, crossing over between repeated sequences that are dispersed throughout the genome (non-allelic HR) could lead to genomic rearrangements, such as translocations, deletions, amplifications and inversions (**Figure 3D**). These models account for genome rearrangements responsible for different human pathologies, attesting to the existence of these processes *in vivo* (Purandare and Patel, 1997; Chen et al., 2007; Fusco et al., 2012).

HR-MEDIATED GENOME REARRANGEMENTS BY BIR AND NON-ALLELIC HR

In *Saccharomyces cerevisiae*, using an intron-based chromosomal translocation assay, it has been reported that DSB-induced translocation occurs via triparental recombination events. A short homologous sequence in the third chromosome serves as a bridge template for recombination events occurring between two non-homologous chromosomes. These events give rise mainly to reciprocal translocations that require the HR proteins Rad52 and Rad51 and the BIR-specific protein Pol32. Rad59 and Srs2 are also required, although to a lesser extent, whereas KU70 plays no role. These data suggest that BIR-mediated triparental recombination could be a major mechanism for chromosomal translocations in eukaryotic cells (Schmidt et al., 2006; Ruiz et al., 2009). Using a newly designed substrate for the analysis of DSB-induced chromosomal translocation, the group of Aguilera shows that

Mus81 and Yen1 endonucleases promote BIR, thus causing non-reciprocal translocations. These endonucleases, as well as Slx4, promote replication template switching during BIR, thus participate in the generation of complex rearrangements when repeated sequences dispersed throughout the genome are involved (Pardo and Aguilera, 2012).

BIR can also induce genome instability in mammalian cells. It was recently reported that replicative stress induced by the overexpression of cyclin E in human cells led to copy number alteration (CNA). One third of these genome alterations (duplications less than 200 kb) have been attributed to BIR events or to microhomology-induced replication (MMBIR), a BIR-related mechanism (see below). The depletion of Pol D3, which encodes a subunit of pol delta, decreases the frequency of these events. The authors propose that BIR repair of damaged replication forks might explain the presence of segmental genomic duplication in human cancers. The larger amplification (>200 kb) and deletion observed after the overexpression of cyclin E may arise from other repair mechanisms, such as non-allelic HR (Costantino et al., 2014).

Replication fork arrest has also been reported to promote non-allelic HR between repeated sequences. In budding yeast, a reduced level of replicative polymerases, which can potentially alter the progression of replication forks, leads to recombination between an inverted Ty element and translocation (Lemoine et al., 2005, 2008). A more direct connection between fork arrest and HR-mediated genome rearrangements has been established in fission yeast, in which the block of a single replication fork leads to translocation and genomic deletion that results from HR between repeated sequences (Lambert et al., 2005; Iraqui et al., 2012). Such chromosomal rearrangements are a direct consequence of replication restart at unbroken forks by HR and not a consequence of failure in restarting forks and subsequent aberrant processing (Mizuno et al., 2009).

Given the potential role of HR in mediating chromosomal rearrangement, factors that prevent non-allelic HR might thus be considered as factors protecting against homology-mediated genomic instability. For example, increasing the distance between repeated sequences reduced the frequency of non-allelic HR (Lichten and Haber, 1989; Godwin et al., 1994). In fission yeast, CENP-B factors facilitate fork passage across LTR repeats that are prone to fork blockage. In the absence of CENP-B, LTR behaves as an HR hot spot prone to deletion events (Zaratiegui et al., 2011).

HR-INDUCED MUTAGENESIS

Mutagenesis associated with HR was first reported in *E. coli* (Cairns and Foster, 1991; Harris et al., 1994; Rosenberg et al., 1994). Repair of DSBs by HR in *E. coli* is non-mutagenic in unstressed cells, but under stress, switches to a mutagenic mode that is activated by stress responses (Ponder et al., 2005; Shee et al., 2011). This mutagenic repair of DNA breaks requires proteins that mend DSBs by HR, error-prone DNA polymerases, activation of SOS DDR, the controlled general and starvation stress response (RpoS), and a membrane protein stress response (RpoE), that promotes spontaneous DNA breakage in some DNA regions (Gibson et al., 2010). RpoS controls the switch that changes the normally high-fidelity process of DSBR via HR to

an error-prone one. In this pathway, three steps are required: (1) DSB repair initiated by HR proteins (RecBCD, RecA); (2) the activation of SOS upregulates PolIV/DinB error-prone DNA polymerase; and (3) a second stress that activates RpoS, which allows Pol I, II, V, and/or PolII to participate in break repair instead of (or in addition to) the high fidelity DNA polIII (for review Rosenberg et al., 2012). This mechanism limits genetic instability to the stress response and to regions near a DSB, and therefore produces localized mutations rather than dispersed mutations. This could be an important evolutionary strategy, both for the minimization of deleterious mutations in cells that acquire a rare adaptive mutation and also for concerted evolution within genes and gene clusters (reviewed in Rosenberg et al., 2012).

Using HO-generated DSBs, it has been shown that mitotic recombination is mutagenic, which has been referred to as break-repair-induced mutation (BRIMs) (Strathern et al., 1995; Rattray et al., 2002; and reviewed in Abdulovic et al., 2006). Both error-prone DNA synthesis associated with DSB repair and stretches of ssDNA might account for BRIMs. During DSB repair, the DNA-end-resection machinery generates intermediates containing ssDNA that are highly sensitive to mutations due to the activity of the trans-lesion synthesis DNA polymerase Zeta (Yang et al., 2008). In addition, it has recently been shown that the DNA synthesis step during elongation of the invading strand is highly mutagenic in *Saccharomyces cerevisiae*, with the mutation rate increasing by up to 1400-fold, and exhibits a mutation signature (primarily microhomology-mediated inter-strand template switching). These mutations result from errors that are made by Polδ and Pole (Hicks et al., 2010). Importantly, HR can be mutagenic even when involving a long tract of DNA synthesis. Indeed, BIR, one of the HR-type processes that are thought to restart replication forks, duplicates DNA over a long distance, even to the end of the chromosome arm, by establishing a replication fork-like structure (**Figure 1B**). Strikingly, in *Saccharomyces cerevisiae*, DNA synthesis that is induced through BIR is highly inaccurate over the entire path of the replication fork. The high level of mutation results from the combinatorial effects of an increase of the nucleotide pool induced by the DDR, the uncoupling of DNA synthesis with mismatch repair, and the exposure of ssDNA (Deem et al., 2011). Recently, BIR has been proposed to proceed via a migrating D-loop mediated by the helicase Pif1. The migration of the D-loop results in the extrusion of the synthesized DNA and the exposure of a long stretch of ssDNA, which can become a hot spot for lesions leading to mutations (Saini et al., 2013; Wilson et al., 2013). In support of this hypothesis, BIR-induced mutations are largely dependent on Pif1 (Saini et al., 2013; Wilson et al., 2013).

One essential role of HR is to reactivate arrested replication forks. In *Schizosaccharomyces pombe*, this process is error-prone. As mentioned above, replication restart by HR mediates non-allelic HR. More surprisingly, it also leads to small deletions and duplications flanked by micro-homology. Indeed, replication forks restarted by HR are associated with error-prone DNA synthesis, liable to template switch events at micro-homologies (Iraqui et al., 2012). When progressing across small inverted repeats or palindromes, forks recovered by HR are prone to generate large chromosomal inversions (Mizuno et al., 2013).

ANTI-HR MECHANISMS FOR PROTECTION AGAINST GENETIC INSTABILITY AND CELL TOXICITY

One mechanism avoiding potential genetic instability promoted by HR is to orientate it to equal SCEs, while unequal SCEs are mutagenic (see **Figure 3B**). Indeed, sister chromatids are identical, thus GC cannot transfer mutation and CO will not have any genetic impact. This is done by associating two processes (as discussed above): (1) restriction of HR in S and G2 phase and (2) the cohesion of the sister chromatids.

Excess HR can also lead to the accumulation of HR intermediates, which generates genomic instability and cell death (Gangloff et al., 2000). Thus, HR is a double-edged sword; on the one hand, it protects against genetic instability, but on the other hand, it can trigger cell lethality as well as profound genomic rearrangements and point mutations. Therefore, the HR process should be tightly controlled to avoid unnecessary HR events. Helicases, by destabilizing abortive HR intermediates, protect against the genomic instability generated by HR (reviewed in Barber et al., 2008; Chu and Hickson, 2009; Bernstein et al., 2010). Additionally, it has been proposed that restricting the initiation of unscheduled HR can also prevent against the accumulation of such toxic HR intermediates. In mammalian cells, this protective role against excessive HR initiation has been proposed for p53, Bcl-2, and AKT1 (Bertrand et al., 2004; Plo et al., 2008; Guirouilh-Barbat et al., 2010; Laulier et al., 2011a; Laulier and Lopez, 2012).

Of note, the fact that protective systems have evolved to counteract excess HR underlines the potential risks of this pathway.

RELATIONSHIPS WITH CHROMOTHRIPSIS AND KATAEGIS

The classical theory of cancer development proposed that cells gradually and randomly accumulate mutations and rearrangements that increase their survival (reviewed in Stratton et al., 2009). However, recent studies have revealed that critical aspects of cancer development can occur on a much shorter timescale. In a process called chromothripsis (from the Greek chromos for chromosome and thripsy, shattering into pieces), tens to thousands of genomic rearrangements occur in one cellular crisis (Berger et al., 2011; Stephens et al., 2011). In kataegis, mutations accumulate in hotspots of hundreds of bases to megabases in a single cell cycle (Nik-Zainal et al., 2012; Roberts et al., 2012). Interestingly, both processes are linked to DSB repair events.

In chromothripsis, cells undergo tens to thousands of genomic rearrangements clustered into discrete subchromosomal territories, as first described in a small set of tumors (Berger et al., 2011; Stephens et al., 2011) and subsequently observed in a wide variety of tumors (Kloosterman and Kuipers, 2011; Magrangeas et al., 2011; Lapuk et al., 2012; Molenaar et al., 2012; Rausch et al., 2012). What causes such a dramatic remodeling of the genome is still unknown. However, the implicated regions are sharply circumscribed and this suggests that the original DNA damage occurs during mitosis when DNA is highly condensed. Although several mechanisms have been suggested to explain the clustered rearrangements, the most plausible cause is replicative stress on regions difficult to replicate (e.g., fragile sites). In particular, replication intermediates that do not expose long stretches of ssDNA and therefore do not activate the checkpoints allow cells to enter mitosis in their presence (Chan et al., 2009). A recent

study suggested that chromosome shattering might arise from an error in mitotic chromosome segregation that leads to the production of micronuclei (Crasta et al., 2012). These micronuclei are at high risk for the integrity of the genome. First, they exhibit a defective DDR and delayed or defective DNA repair (Terradas et al., 2009, 2012; Crasta et al., 2012). Second, most micronuclei replicate more slowly than the major nucleus and therefore most micronuclei are still replicating when the major nucleus is already in the G2 phase (Crasta et al., 2012). Finally, entry in mitosis when the micronucleus is still replicating is associated with a massive induction of DSBs (Crasta et al., 2012).

The DNA repair machinery then reassembles the chromosomal pieces in a disordered fashion (see example in **Figure 4A**). The possible mechanisms of chromosome reassembly first implicated NHEJ and A-EJ because the junction sequences exhibited tracts of microhomology, as well as insertions or

deletions of variable sizes (Rausch et al., 2012; Stephens et al., 2012). However, these mechanisms can account for the loss of genetic information but not for amplification of some genomic regions (Magrangeas et al., 2011; Rausch et al., 2012; Stephens et al., 2012). Replication-based repair pathways are more plausible, accounting for both genomic gains and losses. A hybrid of replication-independent mechanisms and replication-dependent processes has been proposed to explain the complex rearrangements found in chromothripsis, the MMBIR (microhomology-mediated break induced replication) (**Figure 4B**) (Hastings et al., 2009; Liu et al., 2011) associated with a specific mechanism linked to replication block, FoSTeS (for Fork Stalling and Template Switching) (Lee et al., 2007; Zhang et al., 2009). These processes begin with the conversion of a DSB (or a replication fork stall) in a ssDNA 3' stretch. This free 3'DNA end can then anneal using a region of micro-homology on a ssDNA region exposed on an

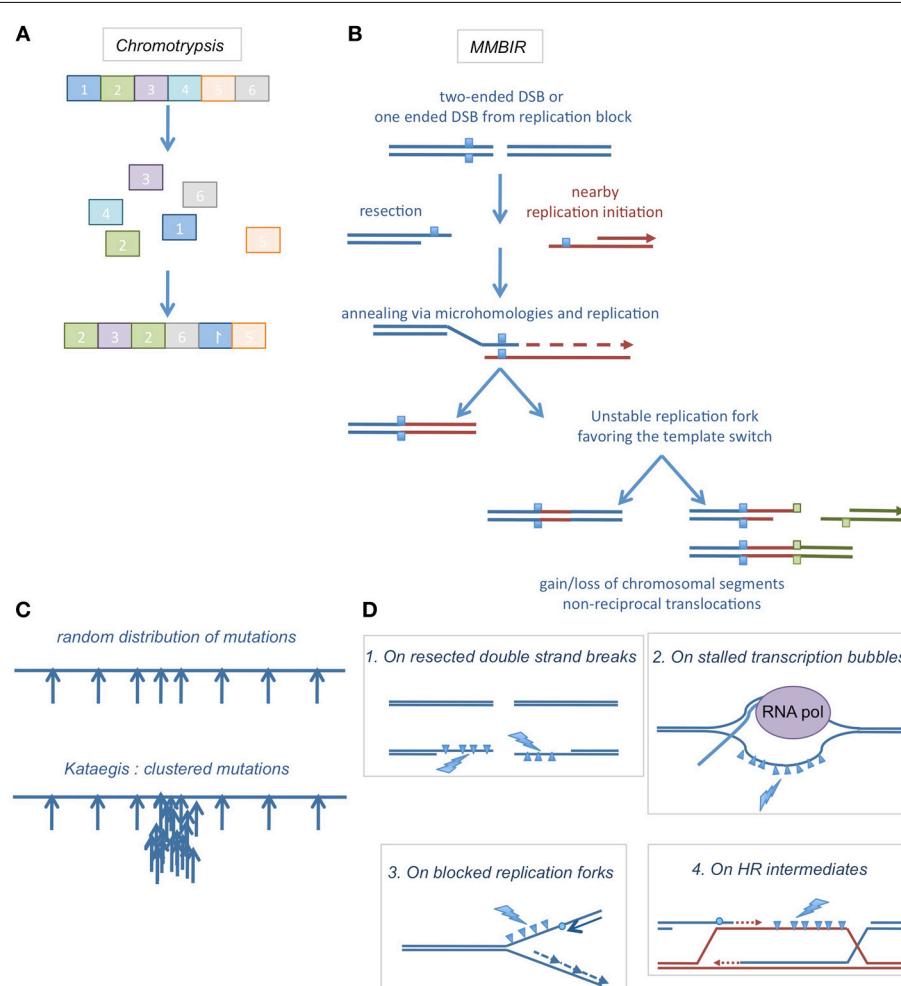


FIGURE 4 | (A) Chromothripsis. Chromosomal shattering into pieces and abnormal re-ligation events, resulting in intra- or inter-chromosomal rearrangements. **(B)** A suggested model for chromothripsis occurrence, the MMBIR (microhomology mediated break induced replication). A DNA double strand end is resected to generate a 3' overhang that will anneal with microhomologies elsewhere in the genome to initiate replication. This mechanism can lead to more complex rearrangements if it is coupled to

multiple cycles of template switches. **(C)** Kataegis. When mutations are expected to be distributed randomly in the genome (upper cartoon), clustered mutations were found in the genomes of several cancers (lower cartoon). **(D)** Where kataegis occurs. These clustered mutations were at least in part correlated with the action of DNA deaminases of the APOBEC family, which deaminate cytosines on ssDNA areas found on resected DNA ends (1), stalled transcription bubbles (2), blocked replication forks (3), or HR intermediates (4).

adjacent replication fork. Replication can then occur. However, such replication forks are weakly processive and can undergo several rounds of template switching, generating complex rearrangements with deletions, amplifications and non-reciprocal translocations. The use of this low fidelity repair process to manage the high level of DSBs generated during chromothripsy could be explained by the overwhelming of reliable repair processes and DDRs. It is worth mentioning that not all chromothripsy events are explainable by FoSTeS or MMBIR; some of them might be the result of chromosome shattering followed by NHEJ or A-EJ.

In 2012 has been reported the occurrence of somatic localized mutation hotspots in tumor genome, called kataegis (from the Greek for thunderstorm) (Nik-Zainal et al., 2012; Roberts et al., 2012). This mechanism was then observed in a broad range of cancers (Alexandrov et al., 2013). In kataegis, mutations accumulate rapidly at somatic mutation hotspots (**Figure 4C**) at a critical step of tumorigenesis. Several mutation signatures were identified, particularly mutations on guanines and cytosines.

The mutation pattern matched the signatures of the RNA- and DNA-editing deaminases of the AID/APOBEC family that act on ssDNA molecules. Indeed these enzymes deaminate cytosines and generate uracils that are a substrate for Base Excision repair, generating abasic sites, causing C-to-T-transitions or driving polymerase eta misincorporations. Before kataegis was described, genome sequencing studies had revealed that many cancers have somatic mutations dominated by C-to-T transitions (Sjöblom et al., 2006; Greenman et al., 2007; Jones et al., 2010; Berger et al., 2011; Kumar et al., 2011; Parsons et al., 2011; Stransky et al., 2011; Taylor et al., 2013) and that overexpression of APOBEC1 was associated with cancer development (Yamanaka et al., 1995) when overexpression of APOBEC3A induced genomic damage and mutations (Stenglein et al., 2010; Landry et al., 2011; Suspene et al., 2011). The implication of APOBEC deaminases in kataegis was validated by several groups in yeast models (Taylor et al., 2004; Chan et al., 2012; Roberts et al., 2012) and in human cells (Burns et al., 2013), where overexpression of APOBEC3B was correlated with an elevated level of mutations in breast tumors and cell lines. Knockdown experiments showed that endogenous APOBEC3B was responsible for increased mutation frequencies and C-to-T transitions when APOBEC3B overexpression induced DNA damage and C-to-T mutations in human cells.

As mentioned above, AID/APOBEC enzymes deaminate only cytosines in ssDNA. It was therefore proposed that these deamination reactions could occur on stabilized ssDNA stretches formed on stabilized transcription bubbles or after the occurrence of DSBs or replication fork blockage (**Figure 4D**). In the last case, the uncoupling between helicases and polymerases generates and stabilizes long patches of ssDNA.

Interestingly these strand coordinated clusters of mutated cytosines or guanines were often localized next to chromosome rearrangement breakpoints and extended up to 200 kb (Roberts et al., 2012) suggesting that they were correlated to the occurrence of DSB and DSB repair pathways generating ssDNA stretches, like HR (see **Figure 1**). The correlation between DSB induction and kataegis was confirmed in yeast treated with alkylating agents (Roberts et al., 2012) or even more directly, in yeast where DSB

were induced by the meganuclease I-SceI (Taylor et al., 2013): In these studies, the authors observed a strand bias in the mutations observed. Cytosines were preferentially mutated on the 5' side of a DSB and guanines on the 3' side of the DSB. As resection only occurs in the 5' to 3' direction, this pattern in mirror was correlated to the generation of ssDNA stretches in Homology directed repair. It is noteworthy that HR is not the only mechanism leading to ssDNA stretches that are a template for kataegis; uncoupled replication forks that expose long stretches of ssDNA are also a template for deaminases (Roberts et al., 2012).

The association of the timescale between kataegis and chromothripsy suggests that both could occur simultaneously at certain chromosomal regions, resulting in an even more catastrophic event for the cell.

THE IMPORTANCE OF BEING VERSATILE

HR is versatile because it tolerates limited divergences between the interacting partners. Remarkably, this capacity to modify genetic information has been used by cells to generate beneficial genetic diversity. HR has therefore been implicated in numerous essential biological processes, from molecular evolution to DNA repair and meiotic differentiation, and is also relevant to targeted gene replacement.

At meiosis, HR ensures that allele mixing creates genetic diversity. In chickens, gene conversion of the expression allele with pseudo-genes generates the complexity of the immune repertoire (Reynaud et al., 1987).

In pathogens, antigenic variation is a widely used strategy for immune evasion. Gene conversion is a prominent system for antigenic variation through recombination between one silent copy of a gene and the expressed copy, resulting in the formation of a chimeric gene. Several pathogens, such as *Trypanosoma brucei*, *Anaplasma marginale*, *Borrelia burgdorferi*, *Helicobacter pylori*, and *Neisseria gonorrhoeae*, use this strategy (Palmer and Brayton, 2007; Stockdale et al., 2008; Wisniewski-Dye and Vial, 2008). For example, trypanosomes are coated with a variant surface glycoprotein (VSG). Antigenic variation involves switches in the composition of the VSG coat driven by gene conversion between the expressed allele and an archive of silent VSG genes (Marcello et al., 2007; Morrison et al., 2009). In *Candida albicans*, recombination generates homozygous hyperactive alleles conferring resistance to antifungals (Coste et al., 2006).

HR is a driving-force in the evolution of multi-gene families; crossovers leading to unequal exchanges between sister chromatids are responsible for variation in the repetition of duplicated sequences. During evolution, most duplicated sequences diverge; the genes of one species derived from a common ancestor are paralogs. Due to selective pressure, there are generally fewer divergences between homologous genes of two different species (orthologs) than between their respective paralogs. However, in some families of repeated genes, the divergence between the duplicated units is less significant within one species than when compared to a different species, even one that is evolutionarily close. In this case, the duplicated genes did not evolve independently but instead co-evolved; this phenomenon is called “concerted evolution” (reviewed Arnheim, 1983; Liao,

1999). Gene conversion is the driving force behind homogenization of duplicated sequences, and therefore of concerted evolution. Concerted evolution is a universal biological phenomenon that occurs in bacteria, yeast, plants and animals. Because HR should be tightly controlled, some processes exist to limit it. Indeed, sequence heterologies block gene conversion and should therefore be barriers to concerted evolution; it has been suggested that introns, which can interrupt the length of sequence homology without affecting the function of the encoded protein, can be protective barriers against HR between repeated sequences, thereby favoring the maintenance of the structural organization of the genome (Kourilsky, 1983; Krieger et al., 1992). In this context, it is tempting to speculate that introns are an evolutionary force antagonistic to concerted evolution, directing evolution toward the divergence of repeated sequences.

UP- AND DOWN-REGULATION OF HR IN CANCER

Genetic instability is a hallmark of cancer cells. Both inhibition and stimulation of HR have been reported in tumors or cancer-prone situations. This is consistent with the duality of HR, and this underlines that inhibition as well as stimulation of HR confer increased risks of genetic instability. More precisely, both down- and up-regulation of the recombinase RAD51 affects genomic stability.

For instance, the expression of a non-lethal dominant negative form of RAD51 in cells injected into nude mice favors tumor take and growth (Bertrand et al., 2003). The overexpression of RAD51 stimulates HR (Vispé et al., 1998; Huang et al., 1999; Lambert and Lopez, 2000) and induces a strong chromosome instability (Richardson et al., 2004), underlying the potential risks of excess HR. These data highlight the importance of tight control of the level of HR.

HR DEFECTS ASSOCIATED WITH PREDISPOSITION TO CANCERS

Most of the mutations responsible for familial breast or ovarian cancers affect genes that control HR and/or the replication/HR interface directly or indirectly (Walsh and King, 2007; Walsh et al., 2011). The two genes most often mutated, BRCA1 and BRCA2, are two major players in HR (Moynahan et al., 1999, 2001). This overrepresentation of genes involved in the response to DNA damage and the communication between replication and recombination suggests the importance of these specific metabolic pathways in the etiology of breast cancer and raises the question of characteristics common to the causation of sporadic and hereditary breast cancer. Several studies have reported the hyperactivation of the oncogenic kinase AKT1 in 40–60% of sporadic breast cancers and in 40% of sporadic ovarian cancers (Sun et al., 2001; Yang et al., 2006; Plo et al., 2008). It must be noted that PTEN, one of the genes mutated in familial breast cancer, is an antagonist of AKT1. Several studies have shown connections between AKT1 and responses to DNA damage (for a review, see Guirouilh-Barbat et al., 2010). In particular, overexpression of AKT1 induces the sequestration of BRCA1 and RAD51 in the cytoplasm, leading to the inhibition of HR (Plo et al., 2008; Plo and Lopez, 2009). Taken together, these data underline the importance of HR in protection against breast cancer and reveal the AKT1 signaling

pathway as a missing link between hereditary and sporadic breast cancers.

Other examples of HR inhibition exist in situations of predisposition to cancer. For example, Bc1-2 is an inhibitor of the intrinsic pathway of apoptosis induction, and its activation confers a predisposition for lymphomas. Bc1-2 was initially found to be overexpressed in B cell lymphoma with the recurrent translocation $t_{(14;18)}$, but it is also overexpressed in numerous tumors. Remarkably, overexpression of Bc1-2 leads to the relocalization of BRCA1 in endomembranes (endoplasmic reticulum, mitochondria), resulting in an inhibition of HR (Laulier et al., 2011a; and reviewed in Laulier and Lopez, 2012).

STIMULATION OF HR IN CANCER

Conversely, there are also situations associating a predisposition for tumors and hyper-recombinogenic phenotypes.

For example, in Bloom syndrome, there is a greatly elevated predisposition to spontaneous tumors in all tissues. Bloom syndrome results from the inactivation of the BLM protein, a member of the RecQ helicase family, that plays an important role in the resolution of HR intermediates, in the processing of blocked replication forks, and at the initiation of DNA double strand break repair (Bernstein et al., 2010; Grabarz et al., 2013). Cells from patients afflicted with Bloom syndrome show increased levels of exchange between sister chromatids and hyper-recombination phenotypes (reviewed in Chu and Hickson, 2009).

The tumor-suppressing p53 gene is the most frequently mutated gene in all types of cancers. It has been shown that the p53 protein represses HR; cells deficient in p53 show a hyper-recombination phenotype (for a review, see Bertrand et al., 2004).

The fusion oncogene BCR/ABL derives from the translocation of the cABL gene from chromosome 9 to the BCR gene locus on chromosome 22: Philadelphia chromosome $t_{(9;22)}$. This translocation is present in chronic myelogenous leukemia (CML) patients and in many acute lymphocytic leukemia patients. The BCR/ABL fusion proteins (p230, p210, or p185) exhibit constitutive tyrosine kinase activity. The resistance of BCR/ABL tumors to DNA damage induced by therapeutic drugs depends on the kinase activity of the fusion protein. The expression of BCR/ABL increases the intracellular level of RAD51 protein by different mechanisms (Slupianek et al., 2001). First, signaling from the BCR/ABL src homoglyc-3 (SH3) and SH2 domains stimulates RAD51 transcription via the activation of the signal transducer and activation transcription 5 (STAT5). The transcription of the paralogs RAD51B, RAD51D, and XRCC2 is also stimulated, whereas transcription of RAD51C and XRCC3 is decreased. Second, BCR/ABL inhibits caspase-3 activation and thus RAD51 protein degradation. Indeed, BCR/ABL stimulates HR between tandem repeat sequences. Additionally, BCR/ABL interacts with RAD51 and results in a high level of constitutive Tyr315 phosphorylation. This Tyr315 phosphorylation and RAD51-dependent HR seem to control resistance to cisplatin and mitomycin C (Slupianek et al., 2001). BCR/ABL expression inhibits DNA-PK activity, which is involved in non-homologous end joining, a competitor pathway to HR for DNA DSB repair (Deutsch et al.,

2001). This suggests that the regulation of the balance between HR and NHEJ can be modified by BCR/ABL.

CONCLUSIONS

HR: A DOUBLE-EDGED SWORD

Regulation of HR should permit the maintenance of genomic stability, allowing genetic diversity but avoiding genetic instability. Depending on the structure of the interacting DNA partners, GC and CO intrinsically possess the capacity to generate genetic variability/instability. In addition to cell cycle regulation, which inhibits HR in the G1 phase and restricts it at the S-G2 phase (during which the sister chromatids are generated) and the tight cohesion of the sister chromatids that orientates exchange to equal SCE, several additional mechanisms repress HR: mismatch repair, helicases, and p53. Defects in these systems are associated with genome instability and cancer predisposition. The fact that living organisms develop strategies to repress HR underlines the potential dangers of HR excess. Indeed, excess HR does generate mutagenesis and genomic rearrangements. These capacities have been used by cell to generate beneficial genetic diversity, but conversely, many pathological rearrangements are explained by accidental HR.

Strikingly, ablation of replication origins in *Archaea* bacteria results in faster growth thanks to the initiation of replication by HR (Hawkins et al., 2013). This raises the question as to why organisms use replication origins to duplicate the entire genetic material, instead of HR. Considering the potential risks of HR both for the accuracy of DNA replication and for genomic architecture, the choice of replication origins should ensure a more stable and accurate duplication through generations; note that this should allow for the maintenance of the minimum common genomic structure defining a given species. In contrast, HR, especially CO, would lead to highly rearranged DNA in offspring, resulting in genetic separation between ancestors and progeny. While genome modification is a driving force for evolution giving opportunity to generate individual genetic diversity, an intergenerational maintenance of the genome should facilitate speciation.

ACCURACY OF HR vs. NHEJ: THE WORLD TURNS UPSIDE DOWN

In many scientific reports (publications, reviews, thesis dissertations, conferences), HR is claimed to be error-free, whereas NHEJ is said to be error-prone. However, the two processes share similarities:

- Both HR and NHEJ are required for genome stability maintenance.
- Both are involved in processes generating genome diversity.
- Both can generate genome rearrangements.
- In both cases, the structure of the DNA molecules determines the final product.

However, they also show differences:

- In contrast with the common view, HR contains the intrinsic capacity to modify genetic material through GC and CO (this has been used to generate genetic diversity in meiosis or V(D)J recombination in chicken) and by promoting

error-prone DNA synthesis, while NHEJ is not intrinsically error-prone and can join fully complementary DNA ends mainly in a faithful manner (for review see Betermier et al., 2014).

Therefore, HR, which can generate genetic alteration, should be tightly control to limits its potential danger and to lead to accurate outcomes. However processes aiming at generating genetic diversity take advantage of these intrinsic capacities of HR.

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BRCA1 in the DNA damage response and at telomeres

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Mutations of the breast and ovarian cancer susceptibility gene 1 (*BRCA1*) account for about 40–45% of hereditary breast cancer cases. Moreover, a significant fraction of sporadic (non-hereditary) breast and ovarian cancers exhibit reduced or absent expression of the *BRCA1* protein, suggesting an additional role for *BRCA1* in sporadic cancers. *BRCA1* follows the classic pattern of a highly penetrant Knudsen-type tumor suppressor gene in which one allele is inactivated through a germ-line mutation and the other is mutated or deleted within the tumor. *BRCA1* is a multi-functional protein but it is not fully understood which function(s) is (are) most important for tumor suppression, nor is it clear why *BRCA1*-mutations confer a high risk for breast and ovarian cancers and not a broad spectrum of tumor types. Here, we will review *BRCA1* functions in the DNA damage response (DDR), which are likely to contribute to tumor suppression. In the process, we will highlight some of the controversies and unresolved issues in the field. We will also describe a recently identified and under-investigated role for *BRCA1* in the regulation of telomeres and the implications of this role in the DDR and cancer suppression.

Keywords: breast cancer susceptibility gene 1, DNA damage response, telomeres, ataxia-telangiectasia mutated, homology-directed repair, base excision repair, DNA damage signaling

INTRODUCTION

The breast and ovarian cancer susceptibility gene 1 (*BRCA1*) on chromosome 17q21 was identified and cloned in 1994 by Miki et al. (1994), 1 year before the reported cloning of a second breast cancer susceptibility gene (*BRCA2*) on chromosome 13q12-13 by Wooster et al. (1995). The *BRCA1* gene fits the classical Knudsen “two hit” model of a tumor suppressor gene. This model was developed by Dr. Alfred Knudsen, Jr. in 1971 and was first applied to understand the genetics of retinoblastoma, a tumor of the cells of the retina in the eye that occurs in very young children. According to this model, a cell requires two “hits” (mutations), one in each allele of a tumor suppressor gene (e.g., *RB1*, the retinoblastoma susceptibility gene) for a cancer to develop. In hereditary cancers, the first “hit” is a germ-line mutation, which is thus present in all somatic cells. The second “hit” (often the deletion of a portion of the chromosome containing the wild-type allele) occurs only in somatic cells within the target tissue, and leads to cancer. In this model, the inheritance pattern is autosomal dominant (since only one mutant allele is inherited). However, at the molecular level, the tumor exhibits a “recessive” pattern, since both alleles must be inactivated for a tumor to occur. In the case of *BRCA1*, women inherit one mutant allele and one wild-type allele; but in nearly all tumors that develop in *BRCA1*-mutation carriers, the wild-type allele is lost (Merajver et al., 1995), leaving no functional *BRCA1* in the tumor cells.

Although inherited *BRCA1*-mutations account for a very small proportion of all breast cancers (2.5–5%), a significant proportion of the much larger group of sporadic (non-hereditary) breast cancers (30–40%) exhibit absent or significantly reduced levels of *BRCA1* protein, suggesting that loss of *BRCA1* function whether

by epigenetic silencing, mutation, or other mechanisms is a common component in the pathogenesis of sporadic breast cancer (Rice et al., 1998; Taylor et al., 1998; Wilson et al., 1999; Esteller et al., 2000; Staff et al., 2003). Consistent with the Knudsen model, inactivating mutations of both *BRCA1* alleles are uncommon in sporadic breast cancers, since the probability of two acquired hits in a somatic cell is much lower than that of a second hit in a cell that has already acquired the first hit by inheritance.

THE HUMAN *BRCA1* GENE AND PROTEIN

The *BRCA1* gene contains 24 exons, 22 of which are coding exons and 2 of which are non-coding (Miki et al., 1994). Exon 11 is the largest exon and encodes about 60% of the protein. The *BRCA1* protein consists of 1863 amino acids, migrates on SDS-PAGE at a molecular mass (M_r) corresponding to 220 kDa, and does not show significant structural homology to other human proteins with the exception of an N-terminal RING domain (amino acid 20–64) and a C-terminal acidic domain (TAD). This TAD can mediate transcription when ligated to a suitable DNA-binding domain (Monteiro et al., 1996). The C-terminal TAD of *BRCA1* contains a tandem repeat of 95 amino acids each called a *BRCA1*-associated C-terminal (BRCT) domain that is homologous to similar domains found within various DNA repair and cell cycle checkpoint proteins (Bork et al., 1997) (see Figure 1). The BRCT domains were subsequently found to be phosphoprotein-binding modules that bind to specific phosphoserine- or phosphotyrosine-containing motifs and are involved in the processes of DNA damage signaling and repair (Manke et al., 2003). The *BRCA1* RING domain was found to interact with another RING-containing protein

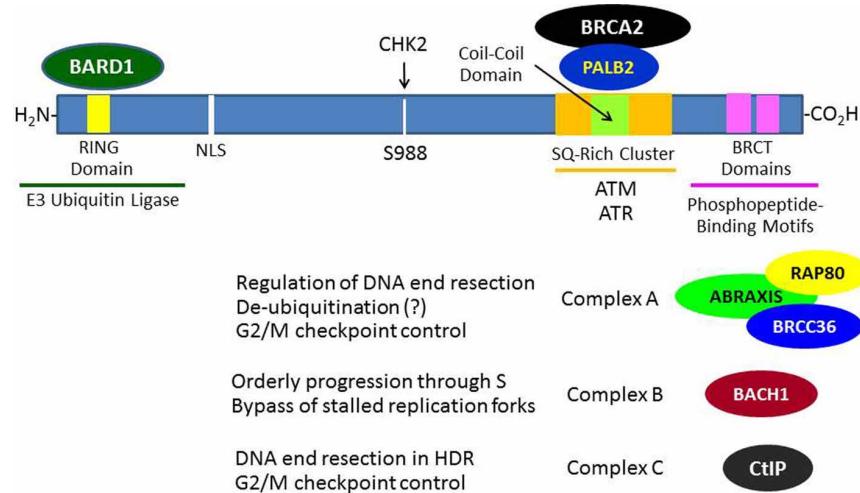


FIGURE 1 | Breast and ovarian cancer susceptibility gene 1 protein interactions that contribute to its role in the DNA damage response. In response to DNA damage, BRCA1 is phosphorylated at various sites by several kinases (e.g., ATM, CHK2, and/or ATR) and forms several different types of complexes that are recruited to the sites of DNA damage through various mechanisms. The roles of these complexes in DNA damage signaling and repair are only partially understood. The formation of these BRCA1

complexes is dependent upon the mutually exclusive interactions of its BRCT domains with phosphorylated motifs within Abraxis, BACH1, or Ctip. BRCA1 functions to recruit BRCA2 to DNA damage sites through an intermediary protein, PALB2 (partner and localizer of BRCA2). The interaction of the BRCA1 N-terminal RING domain with its binding partner BARD1 is required for tumor suppression, since BRCA1-mutations that disrupt this interaction lead to cancer.

BRCA1-associated ring domain 1 (BARD1) protein to mediate an enzymatic function as an E3 ubiquitin ligase (to be discussed below in detail). The BRCA1 protein also contains functional nuclear import and nuclear export signals, suggesting that it may shuttle between the nucleus and cytoplasm, although it seems that most BRCA1 functions occur within the nucleus (Rodríguez and Henderson, 2000).

Breast and ovarian cancer susceptibility gene 1 has been found to regulate the activity of a variety of different transcription factors although BRCA1 is not itself a sequence-specific DNA-binding transcription factor. The usual paradigm is that BRCA1 binds directly to many different transcription factors [e.g., p53, estrogen receptor, progesterone receptor, androgen receptor, STAT1, c-Myc, NF-κB, octamer-binding transcription factor 1 (OCT1), and others], while other portions of the BRCA1 molecule make contact with components of the basal transcription machinery (RNA polymerase II holoenzyme) and/or with components of chromatin remodeling complexes (reviewed in Rosen et al., 2006). In this context, BRCA1 functions as a transcriptional co-regulator that may either stimulate (e.g., p53, androgen receptor, OCT1) or inhibit (e.g., estrogen receptor, progesterone receptor, c-Myc) transcriptional activity. Thus, some BRCA1 functions are linked to the regulation of transcription, although which of these may contribute to tumor suppression remains unclear to date.

ATM-DEPENDENT SIGNALING AND THE DNA DAMAGE RESPONSE

Ataxia-telangiectasia (A-T) is an autosomal recessive hereditary disorder characterized by neurodegeneration (including cerebellar ataxia), immunodeficiency, predisposition to develop cancer, skin abnormalities, and increased sensitivity to ionizing radiation

(IR). A-T is due to mutation of the ATM (A-T mutated) gene, the protein product of which functions as a master regulator of the DNA damage response (DDR) (Lavin and Kozlov, 2007). ATM-deficient cells exhibit hypersensitivity to IR and defects in DNA damage-responsive cell cycle checkpoints (see below). The prototypic activator of ATM is the DNA double-strand break (DSB) due to IR. In the model shown in Figure 2A, the broken DNA ends are recognized by the MRN complex of three proteins [MRE11-RAD-50-Nijmegen breakage syndrome 1 (NBS1)], which functions as a DNA damage sensor and translocates to the site of the DSB (Lee and Paull, 2005). ATM normally exists as an inactive dimer which is maintained in that state by the protein serine/threonine phosphatase PP2A (Bakkenist and Kastan, 2003; Goodarzi et al., 2004). In response to DNA damage, PP2A dissociates from ATM, allowing autophosphorylation on S1981 (and several other residues) and conversion to an active monomer, which is facilitated by physical contact between ATM and the MRN complex at the site of the DSB.

In the context of ATM activation at the sites of DSBs, BRCA1-associated protein required for ATM activation-1 (BAAT1) serves to prevent the premature dissipation of ATM activity by binding to the activated ATM protein and preventing the premature dephosphorylation of ATM at serine-1981 by PP2A (Aglipay et al., 2006). Further activation of ATM is mediated by the chromatin-binding acetyltransferase TIP60 (Sun et al., 2010). TIP60 targets ATM by binding to trimethylated histones near the DSB and acetylating ATM within its PIKK regulatory domain (PRD), which lies adjacent to its kinase domain.

The scheme described above and illustrated in Figure 2A constitutes the classical activation mechanism for ATM in the setting of DNA damage. Recent studies indicate a second mechanism

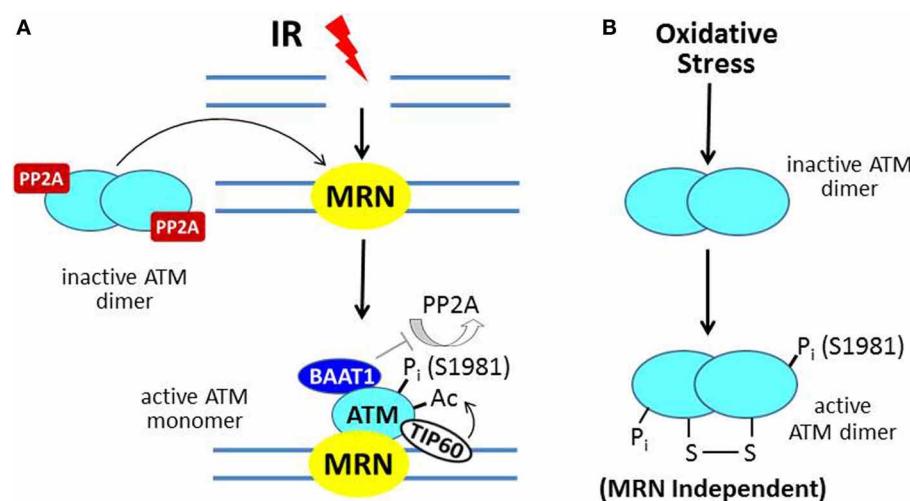


FIGURE 2 | ATM activation by ionizing radiation (IR) vs. oxidative stress. (A) In IR-induced activation, the MRN complex, a DNA damage sensor, is recruited to DSBs; and MRN then recruits ATM. In undamaged cells, ATM is a dimer held in the inactive state by PP2A. After IR, PP2A dissociates from ATM, allowing autophosphorylation on S1981 and conversion to a monomer at the MRN/DSB site. The protein

BAAT1 binds to activated ATM and prevents dephosphorylation by PP2A. Another step in ATM activation involves binding of TIP60 to chromatin near the DSB and acetylation of ATM, which is required for its full activation. (B) In response to oxidative stress, ATM is directly oxidized, forming a disulfide-linked dimer, which is phosphorylated on S1981 and activated.

for ATM activation due to oxidative stress (Guo et al., 2010; Ditch and Paull, 2012). Here, ATM, which also mediates a cytoprotective response to oxidative stress, is activated by a direct mechanism through oxidation of the ATM protein, which does not require the MRN complex (Figure 2B). The result is an active ATM dimer held together by a disulfide linkage that contains two phosphorylated serine-1981 residues.

ATM DOWNSTREAM SIGNALING: RECRUITMENT OF BRCA1 TO DSBs

A large number of different substrates for ATM have been identified (Kastan and Lim, 2000), but herein we will focus on those most closely involved in the recruitment of BRCA1 to the sites of DSBs. In the setting of DNA damage, ATM very rapidly phosphorylates a nearby variant histone (H2AX) on serine-139 (the phosphorylated form of H2AX is known as γ -H2AX), although it is clear that other kinases (e.g., ATM and Rad5-related, ATR) in different contexts can also phosphorylate H2AX (Burma et al., 2001; Wang et al., 2005). Phosphorylated H2AX is recognized by mediator of DNA damage checkpoint protein 1 (MDC1), allowing the recruitment of MDC1 to the sites of DSBs (Stewart et al., 2003; Lee et al., 2005). These events occur very rapidly (within seconds) following the formation of a DSB. MDC1, like H2AX is also a substrate for the ATM kinase. MDC1 serves as a scaffold for the accumulation of other DDR proteins at DNA damage sites and also functions to amplify the DDR (Lou et al., 2006). The proposed mechanism is that MDC1 bound to γ -H2AX can then recruit a second ATM, through the interaction of ATM with the FHA domain of MDC1. This allows phosphorylation of a second H2AX molecule and subsequent recruitment of another MDC1 molecule and so on (Lou et al., 2006; Yan and Jetten, 2008). Besides ATM, MDC1, and γ -H2AX, the MRN complex

is also involved in this amplification process, through a mechanism in which MDC1 is phosphorylated by casein kinase 2 (CK2) and NBS1 binds to MDC1 through its phosphorylated CK2 site (Spycher et al., 2008). These mechanisms allow extension of the DNA damage signal up to 1 Mb upstream and downstream of the original break site and explains why ionizing radiation-induced DNA repair foci (IRIF) can be easily detected by immunofluorescence microscopy (Costes et al., 2010).

Although BRCA1 is phosphorylated relatively rapidly in response to DSBs (see below), its recruitment in large quantities to IRIF is usually delayed (>1 h). Recent progress has elucidated several mechanisms by which BRCA1 becomes localized to IRIF (Kolas et al., 2007; Sobhian et al., 2007; Yan and Jetten, 2008; Strauss et al., 2011; Campbell et al., 2012; Mattioli et al., 2012; Zhang et al., 2012). Two mechanisms are illustrated in Figure 3. In one scheme, the E3 ubiquitin ligase RNF8 binds to MDC1 in a phosphorylation-dependent interaction and along with an associated E2 ubiquitin-conjugating enzyme (Ubc13) ubiquitinates MDC1 on lysine-1977 of MDC1 (Figure 3A). Then RAP80, through its ubiquitin-interacting motif (UIM) binds to ubiquitinated MDC1. RAP80, a component of BRCA1 complex A (Figure 1), recruits BRCA1 to the IRIF through the adaptor protein Abraxis, which interacts directly with BRCA1. This interaction is mediated through binding of the BRCT domain with the pSPXF [phosphoserine-proline-(X = any amino acid)-phenylalanine]. Other components of BRCA1 complex A include BARD1, BRCC36, BRCC45, and NDA1 (MERIT40) (Fong et al., 2009; Wang, 2012). In the second scheme, RNF8 and a second ubiquitin ligase (RNF168) mediate a specific polyubiquitination of a nearby H2AX or H2A molecule through Ubc13, and the ubiquitin chain is recognized by the UIM of RAP80, leading to the recruitment of the BRCA1 complex A (Figure 3B). The possible

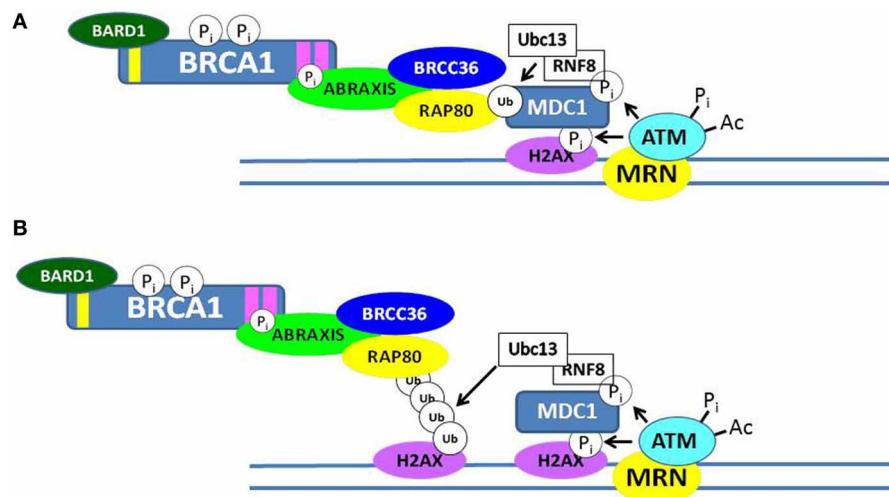


FIGURE 3 | Recruitment of BRCA1 to sites of double-strand DNA breaks.

Two possible mechanisms by which the BRCA1 complex A can be recruited to ionizing radiation-induced foci (IRIF) are illustrated in (A,B). Both involve post-translational modifications of DDR proteins, including phosphorylation and ubiquitination. In (A), the RNF8/Ubc13 complex ubiquitinates MDC1, and

the ubiquitin-interacting motif (UIM) of RAP80 interacts with ubiquitinated MDC1. In (B), RNF8/Ubc13 polyubiquitinates a nearby histone H2AX and the UIM of RAP80 interacts with the ubiquitinated H2AX protein. In each case, phosphorylated Abraxis interacts with the BRCT domain of BRCA1 and the RAP80 protein, thus recruiting BRCA1 to the site of the DSB.

functions of the BRCA1 complexes A, B, and C are considered below. We note here that the recruitment of these complexes to DNA damage sites is mutually exclusive, suggesting that the complexes function during different phases of the cell cycle and/or at different times during the DDR.

BRCA1 FUNCTIONS WITHIN THE DDR

In the context of the DDR, early clues to *BRCA1* gene function came from studies of *Brca1*-deficient fibroblasts and tumors, which exhibited evidence of extensive genomic instability, including a pattern of aneuploidy, centrosomal amplification, and chromosomal aberrations (Tirkkonen et al., 1997; Xu et al., 1999; Weaver et al., 2002). Consistent with these findings, Scully et al. (1997a,b) had reported that: (1) BRCA1 colocalizes with Rad51, a DNA recombinase, in nuclear foci during S-phase; and (2) following DNA damage, BRCA1 became phosphorylated and translocated to PCNA-positive DNA structures containing Rad51, and BARD1. Taken together, these findings suggested a role for BRCA1 as a caretaker gene involved in the monitoring and maintenance of genomic integrity. Other studies indicated that BRCA1-deficient cells showed increased sensitivity to IR (Shen et al., 1998; Scully et al., 1999; Ruffner et al., 2001). Since cell death following IR is mainly due to incomplete repair of DNA DSBs, these findings suggest a role for BRCA1 in the DDR pathways that are activated in response to DSBs.

ROLE OF BRCA1 IN DNA DAMAGE-ACTIVATED CELL CYCLE CHECKPOINTS

Further research suggested specific roles for BRCA1 in response to DNA damage induced by IR. Thus, BRCA1 was found to be required for several DNA damage-responsive cell cycle checkpoints. These checkpoints are activated in response to DNA damage (e.g., DSBs) and function to block cell cycle progression

in order to allow the repair of DNA lesions, so the damage is not propagated and passed on to daughter cells. One such BRCA1-regulated cell cycle checkpoint is the G2/M checkpoint (Xu et al., 2001; Yarden et al., 2002). Here, BRCA1 was found to be essential for the activation of checkpoint kinase 1 (CHK1), a key effector of G2/M arrest (Yarden et al., 2002). Both ATM (A-T mutated) and BRCA1 were found to be required for the IR-induced S-phase as well G2 checkpoints; and a specific phosphorylation of BRCA1 by ATM at serine-1423 was required for activation of the G2 the checkpoint (Xu et al., 2001). BRCA1, as well as the ATR protein, were also found to participate in another G2 cell cycle checkpoint known as the decatenation checkpoint (Deming et al., 2001). This checkpoint monitors the status of chromatid unwinding and delays cell entry into mitosis until the chromatids are sufficiently unwound (decatedenated), in order to prevent chromosomal stress that might lead to aneuploidy or polyploidy.

The DNA damage-induced S-phase checkpoint results in inhibition of replication initiation in response to DNA damage. A defect in this checkpoint results in continued DNA synthesis, also called radioresistant DNA synthesis following IR. This checkpoint was found to require an ATM-dependent phosphorylation of BRCA1 on serine-1387 as well as a functional NBS1 (Xu et al., 2002). In addition to DNA damage-responsive checkpoints, several studies indicate that BRCA1 also regulates the mitotic spindle checkpoint by regulating gene expression associated with orderly progression through mitosis (Wang et al., 2004; Bae et al., 2005). Here, BRCA1 deficiency caused a defect in the spindle checkpoint (which ensures orderly separation of chromatids) as well as a defect in cytokinesis that resulted in accumulation of multinucleated cells. Several recent studies suggest that a CHK2-mediated phosphorylation of BRCA1 (see Figure 1) is required for orderly assembly of the mitotic spindle and proper segregation of chromosomes (Stolz et al., 2010a,b).

Finally, a role for BRCA1 in the IR-induced G1/S checkpoint, which blocks entry of cells containing chromosomal breaks into S-phase, has been demonstrated. Here, in response to IR, ATM phosphorylates BRCA1 on serine-1423 and serine-1524, which allows the efficient ATM-mediated phosphorylation of p53 on serine-15, activation of p53 transcriptional activity, and subsequent expression of the cell cycle inhibitor p21 (Fabbro et al., 2004). In this study, the BRCA1/BARD1 complex was required for the ATM phosphorylation of p53 and subsequent G1/S cell cycle arrest.

ROLE OF BRCA1 IN HOMOLOGY-DIRECTED DNA REPAIR

Double-strand breaks can be repaired by two major pathways: (1) homology-directed repair (HDR; also called homologous recombination); or (2) non-homologous end joining (NHEJ) (Symington and Gautier, 2011). Here, we will consider the role of BRCA1 in HDR, while its putative role in NHEJ will be discussed in the next section. HDR can only occur during S-phase and G2-phase, because the homologous strand of the corresponding sister chromatid is required as a template for repair-related DNA synthesis. This form of DSB repair is usually considered to be error-free, and thus a mechanism for maintenance of genomic integrity (but see below). Moynahan et al. (1999) first described a major role for BRCA1 in HDR based on the finding that a *Brc1*-deficient reporter mouse embryonic stem (ES) cell line failed to accurately repair a chromosomal DSB created by the I-Sce 1 endonuclease. In related studies, the same group demonstrated that BRCA2 was also required for HDR and that the defect in HDR in *Brc1*-deficient cells could be corrected by either expression of a wild-type BRCA1 transgene or correction of one mutated *Brc1* allele through gene targeting (Moynahan et al., 2001). While these studies definitively establish a role for BRCA1 in HDR, they do not address its biochemical function in HDR. One clue to this function is the demonstration of a requirement for an ATPase-competent RAD51 protein for HDR, which was not surprising since RAD51 is the mammalian homolog of the bacterial DNA recombinase RecA (Stark et al., 2002). It has been suggested that HDR is the major tumor suppressor function for both BRCA1 and BRCA2, since a deficiency in HDR leads to increased levels of NHEJ and single-strand annealing (SSA), both of which are error-prone processes that lead to genomic instability.

While the process of homologous recombination has been extensively investigated over several decades, the role of BRCA1 in this process has not been fully worked out. A review of this process and its potential role in tumor suppression can be found elsewhere (Moynahan and Jasin, 2010). The first step in HDR involves the 5'-3' end resection of DNA starting at the site of the DSB. This resection creates a segment of single-stranded DNA (ssDNA) that can then invade the sister chromatid and pair with the complementary DNA strand, allowing the initiation of repair. These resected ends can then be utilized by RAD51, which catalyzes the crossover reaction. In this regard, it is thought that BRCA1 in complex with the CtBP-interacting protein (CtIP; designated “complex C”) (Figure 1) facilitates the end resection by allowing the recruitment of replication protein A (RPA), a ssDNA-binding protein (Sartori et al., 2007; Buis et al., 2012; Escribano-Díaz et al., 2013). The phosphorylation of CtIP that is required for its recognition of and binding to the BRCT domains of BRCA1 is mediated by

cyclin-dependent kinase 2 (CDK2) and is facilitated by MRE11, a component of the MRN protein complex (Buis et al., 2012); and both the MRN complex and CtIP were found to contribute to DNA end resection at the sites of DSBs (Sartori et al., 2007). Complex C also participates in the G2/M cell cycle checkpoint (Yu et al., 2006).

After end resection and recruitment of RPA to the newly created ssDNA, the recombinase RAD51 is recruited to the resected ends. The recruitment of RAD51 is dependent upon other proteins, including RAD54 and BRCA2 (which directly binds multiple copies of RAD51 copies and regulates their activity) (Gudmundsdóttir and Ashworth, 2006). Partner and localizer of BRCA2 (PALB2) is required for the localization of BRCA2 at DNA damage sites and its participation in HDR and, in turn, PALB2 binds directly to BRCA1, suggesting that it functions as an adapter between BRCA1 and BRCA2 during the process of HDR (Sy et al., 2009; Zhang et al., 2009; Buisson and Masson, 2012) (see Figure 1). The final steps in DSB repair by HDR involves the formation two Holliday junctions, which are then resolved without crossover, restoring the DNA to its original condition without sequence abnormalities (Moynahan and Jasin, 2010). It is noted here that while both BRCA1 and BRCA2 function in the HDR pathway, the role of BRCA1 in the DDR is broader than that of BRCA2, since BRCA1 also mediates cell cycle checkpoints.

FUNCTIONS OF BRCA1 COMPLEXES A, B, AND C

Although HDR is generally considered to be an error-free process, an aberrant error-prone form of homologous recombination called “hyper homologous recombination” (HHR) has been described (Harris and Khanna, 2011; Dever et al., 2012). HHR was observed in the presence of mutant forms of BRCA1 (e.g., M1775R) that disrupt the interaction of the BRCT domain with phosphopeptide sequences or when components of complex A (Abraxas, RAP80, or BRCC36) were knocked down (Figure 1). It has been suggested that BRCA1 complex A functions, in part, as a de-ubiquitinating complex to limit end resection during the early stages of HDR to prevent excessive accumulation of RAD51 and RPA on the invading DNA strand. Other studies indicate that complex A also participates in the G2/M cell cycle checkpoint and in localizing BRCA1 to IRIF (Kim et al., 2007a,b; Wang et al., 2007a).

The BRCA1 complex B consists of BRCA1 and BACH1 (BRCT helicase; also known as BRIT and FANCJ) and is formed by the interaction of the BRCA1 BRCT with phosphoserine-990 of BACH1, which is part of a pSPXF motif (Cantor et al., 2004; Peng et al., 2006; Kumaraswamy and Shiekhattar, 2007; Gong et al., 2010; Tomimatsu et al., 2012). The function of complex B is not as clear, but it has been suggested that complex B is required for orderly progression through S-phase, including the bypassing of stalled replication forks, and also serves a DNA repair function that is not well defined. Complex C (BRCA1-CtIP-MRN) is formed through the interaction of the BRCA1 BRCT domain with phosphoserine-327 of CtIP, which is also part of a pSPXF motif. This complex is thought to stimulate DNA end resection by MRE11 during DNA repair by HDR (reviewed in Wang, 2012). However, another nuclease, EXO1, may play a more important role in DNA end resection during DSB repair (Tomimatsu et al., 2012). Knockdown of components of complex A or C cause a defect in the

G2/M cell cycle checkpoint, but the defect created by disruption of complex C is more mild than that created by knockdown of complex A. The molecular details of how these complexes function, their precise roles in the maintenance of genomic integrity, and the mechanisms by which they are assembled and disassembled remain to be determined. Additional information on the BRCA1 complexes and their significance can be found elsewhere (Wang, 2012).

ROLE OF BRCA1 IN NON-HOMOLOGOUS END JOINING

Non-homologous end joining involves a very different set of proteins from HDR [e.g., Artemis, XRCC4, DNA polymerase lambda, DNA ligase IV (LIG4), and DNA-dependent protein kinase (DNA-PK)] and, unlike HDR, occurs predominantly during G1 and less so in S-phase or G2 (Lieber, 2010; Dever et al., 2012). HDR cannot occur during G1, because a homologous segment of DNA that can act as a template for repair synthesis is unavailable in G1. The significance of this process is that it can be an error-prone process because of modification of the broken DNA ends, which can result in short or longer deletions. With regard to the DDR, cells defective for NHEJ show hypersensitivity to IR, suggesting that NHEJ is a major pathway for repair of DSBs generated by IR. The literature on the putative role of BRCA1 in NHEJ is unsettled, because several studies suggest a requirement for BRCA1 in NHEJ (Baldeyron et al., 2002; Zhong et al., 2002a,b; Bau et al., 2004), while others find no defect in NHEJ in BRCA1-deficient cells (Moynahan et al., 1999; Wang et al., 2001; Mérél et al., 2002). While the role of BRCA1 in NHEJ remains controversial, a suggested explanation is that there are several forms of NHEJ, including one that is error-prone and another that is relatively precise; and BRCA1 only promotes the precise end joining (Durant and Nickoloff, 2005; Gudmundsdottir and Ashworth, 2006). The presumed mechanism is that BRCA1, when bound to DNA, inhibits the nuclease activity of MRE11 or the MRN complex, thus limiting DNA end resection (Durant and Nickoloff, 2005).

The participation of BRCA1 in the choice and execution of DSB DNA repair pathways is illustrated in **Figure 4**. Recent studies have identified a cell cycle-dependent mechanism that underlies the DNA DSB repair pathway choice. p53 Binding protein 1 (53BP1), when phosphorylated by ATM, binds to RAP1-interactibg factor 1 (RIF1) and recruits RIF1 to DSB sites, where it inhibits 5' end resection required for HDR, thus promoting the NHEJ pathway. In contrast, BRCA1 promotes 5' end resection and thus HDR (Escribano-Díaz et al., 2013; Feng et al., 2013). BRCA1 expression is normally low in G1 and increases significantly during S-phase and G2. The accumulation of BRCA1 during S and G2 counteracts the ability of 53BP1-RIF1 to stimulate NHEJ. In contrast, when BRCA1 levels are low during G1, 53BP1-RIF1 accumulate at DSBs unopposed by BRCA1, resulting in NHEJ being the predominant pathway for DSB reverse during G1.

BRCA1/BARD1 AND ITS UBIQUITIN LIGASE FUNCTION

Breast and ovarian cancer susceptibility gene 1-associated ring domain 1 was first identified as a RING domain protein that interacts and colocalizes with BRCA1 through a RING: RING interaction involving the N-termini of each protein (Wu et al., 1996; Jin et al., 1997) (**Figure 1**). Several cancer-associated point

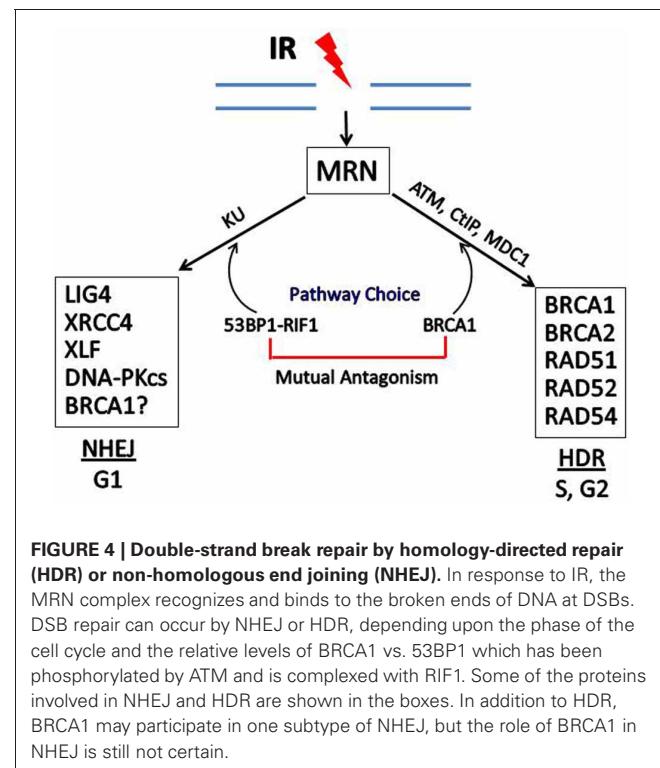


FIGURE 4 | Double-strand break repair by homology-directed repair (HDR) or non-homologous end joining (NHEJ). In response to IR, the MRN complex recognizes and binds to the broken ends of DNA at DSBs. DSB repair can occur by NHEJ or HDR, depending upon the phase of the cell cycle and the relative levels of BRCA1 vs. 53BP1 which has been phosphorylated by ATM and is complexed with RIF1. Some of the proteins involved in NHEJ and HDR are shown in the boxes. In addition to HDR, BRCA1 may participate in one subtype of NHEJ, but the role of BRCA1 in NHEJ is still not certain.

mutations of the RING domain of BRCA1 (e.g., Cys61Gly and Cys64Gly) disrupted the BRCA1: BARD1 interaction, suggesting that this interaction contributes to BRCA1-dependent tumor suppression. An early functional study suggested that BARD1 in association with CstF-50 plays a role in regulation of RNA processing during transcription by inhibiting polyadenylation (Kleiman and Manley, 1999); and a subsequent study suggested that this function may be linked to DNA repair (Kleiman and Manley, 2001). A significant advance in understanding the physiologic importance of the BRCA1: BARD1 interaction was the finding that the BRCA1: BARD1 heterodimer functions as an E3 ubiquitin ligase and that this ubiquitin ligase activity was abolished by cancer-associated mutations within the BRCA1 RING domain (Brzovic et al., 2001; Hashizume et al., 2001). These findings led to the hypothesis that many of the functions of BRCA1, including its tumor suppressor activity, were due to or required the ubiquitin ligase activity of the BRCA1: BARD1 complex (Baer and Ludwig, 2002). Further study suggested that the BARD1 interaction with BRCA1 is required for HDR of chromosomal breaks (Westerman et al., 2003). This finding coupled to the observation of cancer-associated BRCA1-mutations that disrupt the association of BRCA1 with the E2 ubiquitin-conjugating enzyme UbcH5 (Morris et al., 2006) and the finding that cancer-associated RING domain mutations of BRCA1 that disrupt the ubiquitin ligase function cause hypersensitivity to IR (Ruffner et al., 2001). These considerations led to a great interest in identifying the *in vivo* targets of the BRCA1: BARD1 ubiquitin ligase (Wu et al., 2008).

Then, in 2008, Ludwig and his colleagues generated an isogenic set of murine ES cells that expressed either wild-type *Brcal* or a mutant *Brcal* (I26A) that lacks E3 ubiquitin ligase activity but

retains the ability to bind to Bard1 (Reid et al., 2008). Surprisingly, not only were the *Brcal* I26A mutant ES cells viable, but they also exhibited normal sensitivity to the DNA cross-linking agent mitomycin C, formed RAD51 foci in response to IR, and exhibited wild-type rates of HDR (Reid et al., 2008). These findings challenged the prevailing view that the BRCA1: BARD1 E3 ubiquitin ligase activity, the only known enzymatic function of BRCA1, was required for most major functions of BRCA1 thought to be critical for tumor suppression. In a subsequent study, the same investigators demonstrated that transgenic mice homozygous for mutant *Brcal* I26A targeted to specific tissues (e.g., pancreas or mammary gland) suppressed tumor formation to the same degree as wild-type *Brcal*; whereas a *Brcal*-mutation of the BRCT domain that abrogated phosphoprotein-binding (S1598F) conferred a high rate of tumor formation in the same genetic models (Shakya et al., 2011). The investigators concluded that the ubiquitin ligase function of BRCA1 was dispensable for tumor suppression, while the recognition of phosphoproteins by the BRCT domains of BRCA1 was essential for suppression of tumor formation.

These findings have still not finally settled the question of the role of the BRCA1 ubiquitin ligase function in tumor suppression. For example, it was recently reported that BRCA1 normally plays a major role in repressing satellite DNA (Zhu et al., 2011). Satellite DNA consists of long stretches of non-coding DNA characterized by tandemly repeated sequences; and it is a major component of heterochromatin. In this study, BRCA1 deficiency led to the loss of transcriptional repression of satellite DNA in mouse mammary tumors, human breast cancers, and cultured cells and to loss of ubiquitinated histone H2A within the satellite repeats. Furthermore, BRCA1 was shown to bind to satellite DNA and ubiquitinate H2A. The BRCA1-deficient phenotype was reversed by ectopic expression of an H2A-ubiquitin protein. Conversely, this phenotype (including genomic instability associated with defects in HDR and cell cycle checkpoints) was reproduced by the ectopic expression of satellite RNA. The authors' conclusion that most BRCA1 tumor suppressor functions are due to its role in the maintenance of heterochromatin structure (Zhu et al., 2011) is inconsistent with the idea that the BRCA1 ubiquitin ligase function is dispensable for tumor suppression (Shakya et al., 2011).

Indirect evidence regarding BRCA1 tumor suppressor function comes from a study by Gayther et al. (1995) who described a genotype-phenotype correlation with location of the mutation within the *BRCA1* gene, in *BRCA1* breast and/or ovarian cancer families. Examination of the ratio of breast/ovarian cancer revealed that mutations that mapped to the N-terminus of the BRCA1 protein (including missense mutations and protein-truncating mutations that deleted the BRCT domains) exhibited a higher ratio of breast/ovarian cancers than did mutations mapping to the C-terminal portion of the *BRCA1* gene. These findings suggest that BRCA1 proteins missing the BRCT domains (and thus defective for HDR) can still suppress development of ovarian cancer. Other interpretations of these data are possible, but they do suggest differences in the mechanisms for development of breast vs. ovarian cancer in *BRCA1*-mutation carriers.

Moreover, the idea that *BRCA1* deficiency causes cancer solely due to genomic instability associated with the loss of HDR and cell cycle checkpoints does not account for the limited spectrum of

tumor types observed in *BRCA1*-mutation carriers. Thus, a study of nearly 700 *BRCA1* families indicates that breast and ovarian cancers are by far the most common, while there is a higher than expected risk in several additional hormonally related cancers, including cervical cancer, uterine cancer, and prostate cancers in younger men (Thompson et al., 2002). These considerations suggest that *BRCA1* exerts another function(s), perhaps endocrine-related, that collaborates with its role in maintenance of genomic integrity to explain why *BRCA1*-mutations lead to a specific set of tumor types. Thus, *BRCA1* was found to inhibit estrogen receptor activity, both in cultured cells and mouse models; and accumulating evidence suggest that *BRCA1*-related tumorigenesis is a hormonally responsive process, both in mice and humans (reviewed in Rosen et al., 2005).

Finally, the finding that the ubiquitin ligase function of the *BRCA1*: BARD1 is not required for HDR or tumor suppression in the mouse raises the additional question of why missense mutations of *BRCA1* that disrupt the *BRCA1*: BARD1 interaction (e.g., Cys61Gly) lead to cancer in humans and in mice (Drost et al., 2011). These considerations would suggest that the *BRCA1*: BARD1 interaction may have another ubiquitin ligase-independent function that is essential for tumor suppression. Interestingly, BARD1 is itself a tumor suppressor, mutations of which have been linked to breast, ovarian, and endometrial cancers (Ghimenti et al., 2002; Sauer and Andrulis, 2005). However, curiously, some of the cancer-associated mutants of BARD1 do not alter the function of BARD1 in HDR (Laufer et al., 2007).

ROLE OF p53 IN *BRCA1*-DEPENDENT TUMORIGENESIS

As noted above, *BRCA1* can interact directly with p53 and stimulate its transcriptional activity. Interestingly, in studies of human *BRCA1*-related cancers, the incidence of p53 mutations (over 80%) is considerably higher than in sporadic breast cancers (25%) (Phillips et al., 1999; Holstege et al., 2009). Studies of *Brcal* knockout mice revealed early embryonic lethality, usually by day 7.5 (Hakem et al., 1996). The *Brcal*-/- phenotype was characterized by widespread defects in cell proliferation due, in part, to p53 activation. This phenotype was partially reversed by a p53 or p21 deficiency, resulting in embryonic death at later times (Hakem et al., 1997). It was suggested that p53 was activated due to chromosomal abnormalities created by *Brcal* deficiency, causing p53 activation and p21 expression, resulting in cell cycle arrest or senescence. By the same reasoning, it appears that a p53 mutation is required for *BRCA1*-related breast cancer development because otherwise, chromosomal aberrations due to *BRCA1* deficiency would activate p53, leading to cell cycle arrest, apoptosis, and/or senescence of the tumor cells.

In a recent study, it was found that p53 mediates the nuclear export of wild-type *BRCA1* via a *BRCA1*: p53 protein interaction and possibly, in part, by disrupting the *BRCA1*: BARD1 interaction (Jiang et al., 2011). It was suggested that this mechanism could increase cellular sensitivity to DNA damaging agents such as IR and that loss of p53 function could impair the nuclear export of *BRCA1* in sporadic breast cancers with functional *BRCA1*, resulting in greater resistance to DNA damaging agents. Thus, the functional interaction of *BRCA1* and p53 is quite complex and may influence the molecular pathogenesis of breast cancer, the

DDR of tumor cells, and their sensitivity to DNA damaging agents including chemotherapy drugs and IR.

BRCA1 AND TELOMERES

Telomeres, the ends of chromosomes that contain varying lengths of hexameric DNA repeats (TTAGGG in mammalian cells) are of interest in the context of DNA repair for several reasons: (1) if chromosome ends were recognized as DSBs, it would lead to genomic instability due to end joining and translocations; (2) conversely, telomerase is recruited to internal DSBs, where it could potentially generate a telomere, with disastrous consequences; (3) DNA damage can cause telomere shortening; and (4) telomere shortening can lead to chromosomal instability and cancer development (Günes and Rudolph, 2013; Ribeyre and Shore, 2013). Moreover, tumor cells that do not express telomerase, the enzyme complex that adds TTAGGG to telomeres, utilize alternative lengthening of telomeres (ALT), a method of telomere maintenance that involves DNA recombination and utilizes some of the same DNA repair proteins involved in repairing DSBs (Nandakumar and Cech, 2013). To start out, telomeres are protected from being recognized as DSBs, in part, by a complex of six intrinsic telomeric proteins known collectively as "shelterin" (TRF1, TRF2, TIN2, TPP1, POT1, and RAP1). Three of these proteins directly bind telomeric DNA (TRF1, TRF2, and POT1); and the other three proteins (TIN2, TPP1, and RAP1) do not directly contact DNA but serve to interconnect the three DNA-binding proteins. The shelterin proteins contribute to the formation of the telomere loop (t-loop) at the end of the chromosome and

inhibit the activity of ATM and ATR (Raffaella Diotti and Loayza, 2011). In particular, TRF2 and POT1 have been implicated in the inhibition of ATM and ATR, respectively. Other proteins can bind transiently to shelterin to alter telomere function.

One group of proteins that bind to shelterin is the MRN complex. In this context, the same MRN complex which initiates the repair of DSBs, when recruited to the telomere by TRF2 has been implicated in regulation of telomere length and the telomeric overhang (Lamarche et al., 2010) (Figure 5A). MRN as well as the nuclease Apollo have been implicated in generation of the telomeric overhang (a 3' G-rich single-stranded telomeric extension), which functions in maintaining telomere stability. However, far less is known about how telomeric MRN functions than how MRN functions in the sensing and repair of DSBs. As in the case of the response to DSBs, ATM is required for MRN signaling in the context of a dysfunctional uncapped telomere (Lamarche et al., 2010). In this setting a signaling cascade similar to that induced by DSBs is activated and can result in cell cycle arrest, senescence, or apoptosis.

In addition to MRN, several studies implicate BRCA1 as a regulator of telomere length and stability. In the first study, over-expression of BRCA1 was found to inhibit telomerase enzymatic activity by transcriptionally repressing expression of the telomerase catalytic subunit (telomerase reverse transcriptase, TERT) (Xiong et al., 2003). The mechanism of repression appears to be by inhibiting the ability of the c-Myc oncogene to stimulate TERT expression through the c-Myc E-box within the TERT proximal promoter. As a consequence of telomerase inhibition,

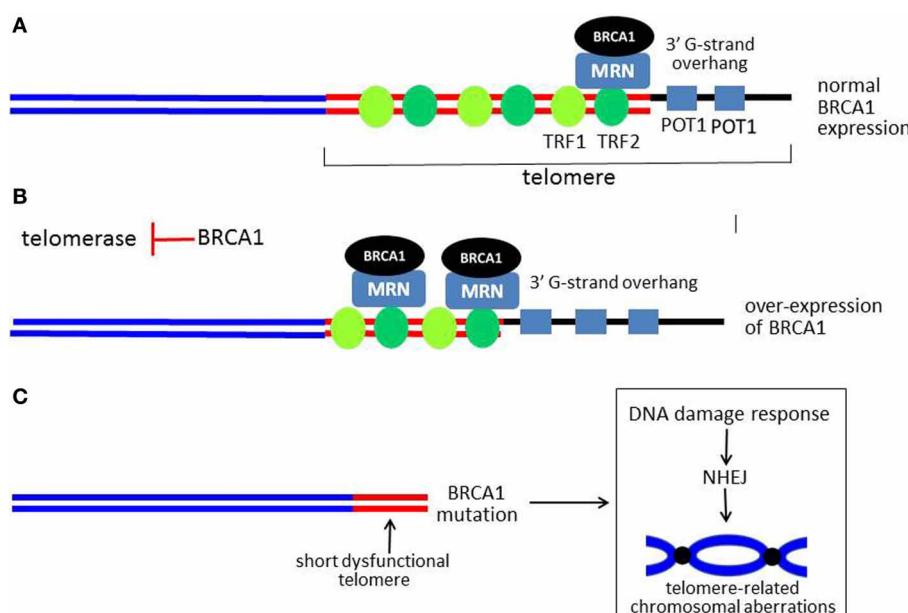


FIGURE 5 | Model for role of BRCA1 in telomere maintenance. (A)

Shows a linear representation of a normal functional telomere. For simplicity, not all of the telomere-associated proteins are shown. BRCA1 is recruited to the telomere by RAD50, a component of the MRN complex, which is also present at the telomere. When BRCA1 is over-expressed, more BRCA1 is present at the telomere. BRCA1 causes overall telomere shortening, but the 3' G-strand overhang is lengthened, as illustrated in

(B). (C) Shows a critically short and dysfunctional telomere with little or no 3' G-strand overhang in cells with no functional BRCA1. A DDR is activated with resultant chromosomal aberrations due to end-end fusions and translocations (a dicentric chromosome is illustrated). The G-strand overhang is represented by a thick black line. The thick red lines represent double-stranded telomeric DNA, while the sub-telomeric DNA is shown as blue lines.

over-expression of wild-type BRCA1 but not a cancer-associated mutant (Cys61Gly) caused telomere shortening in several tumor cell lines (**Figure 5B**). Interestingly, despite causing shortening of telomeres to very small sizes (well under 2.0 kb), BRCA1 did not cause inhibition of cellular proliferation, cell cycle arrest, senescence, or apoptosis (Xiong et al., 2003). Surprisingly, significant telomere shortening due to BRCA1 occurred very rapidly (within 2–3 cell doublings), far too fast to be attributable to inhibition of telomerase activity. These findings suggest that BRCA1 causes telomere erosion (degradation) but somehow protects against telomeric dysfunction.

In a second study, it was found that BRCA1 knockdown resulted in increased telomerase activity and significant telomere lengthening in tumor cells (Ballal et al., 2009). Based on telomeric chromatin immunoprecipitation (telomeric ChIP) assays, the presence of BRCA1 on telomeres was documented. BRCA1 was also found to interact and colocalize with shelterin proteins TRF1 and TRF2, in DNA-dependent manner. In further studies, it was found that BRCA1 was recruited to the telomere by RAD50, a component of the MRN complex. Finally, it was found that, like MRN, BRCA1 regulates the length of the 3' G-rich overhang. Thus over-expression of BRCA1 caused lengthening and knockdown of BRCA1 or RAD50 caused a similar degree of shortening of the 3' overhang (**Figure 5B**). These findings suggest that BRCA1 can regulate both telomere length and telomere stability and may mediate some of the effects of the MRN complex on the telomere (e.g., overhang length). These findings are consistent with the observation that cells with no functional BRCA1 exhibit evidence of telomere dysfunction and loss of the capping function, evidenced by very short telomeres and the appearance of chromosomal abnormalities of the type expected from telomere dysfunctions (e.g., dicentric chromosomes due to end-end fusion) (Al-Wahiby and Slijepcevic, 2005; McPherson et al., 2006; Wang et al., 2007b) (**Figure 5C**). In another study, knockdown of BRCA1 in a mammary epithelial cell line caused chromosomal aberrations consistent with telomere dysfunction (e.g., anaphase bridges). In addition to BRCA1 and MRN, defects of other DDR-associated proteins (Ku, DNA-PKcs, and RAD51D) have been linked to the loss of the telomeric capping function (Cabuy et al., 2008).

Finally, in understanding the relationship between telomeres and the DDR, it was mentioned above that telomerase is recruited to DSBs, where under some conditions it can synthesize telomeres at the broken ends of DNA (Ribeyre and Shore, 2013). The discovery of telomeric DNA sequences within the interiors of chromosomes interstitial telomeric sequences (ITS) of rodents and primates has been interpreted to mean that at some time during evolution telomerase was utilized to repair DSBs (Slijepcevic, 2006). Based on mutational analysis, several studies have demonstrated the existence of active mechanisms in yeast to prevent the synthesis of telomeres by the enzyme telomerase at DNA ends of DSBs (Nergadze et al., 2007; Makovets and Blackburn, 2009; Zhang and Durocher, 2010). For example, Mec1 (the ortholog of ATR in yeast) both recognizes DNA ends and inhibits telomerase at DSBs, a mechanism for the preservation of genomic integrity. Two such mechanisms involve Mec1-dependent phosphorylation of Pif1 (a telomerase inhibitor) and Cdc13 (a telomere capping protein) (Makovets and Blackburn, 2009; Zhang and Durocher,

2010). Genetic analysis in *Drosophila* identified ATR-interacting protein (ATRIP) as a factor involved in preventing the formation of telomeres at the sites of DSBs (Beaucher et al., 2012). In mammalian cells, ATR: ATRIP complexes are recruited to ssDNA coated with RPA and are activated by a complex mechanism that is not fully understood (Liu et al., 2011). In this context, ATR: ATRIP complexes have been found to activate a CHK1-dependent checkpoint mechanism during S-phase in response to stalled replication forks (Nam and Cortez, 2011).

OTHER DNA DAMAGE-RELATED FUNCTIONS OF BRCA1

While the best studied DNA damage-related function of BRCA1 is its role in the repair of DSBs, roles for BRCA1 in other DNA repair processes have been reported. Thus, BRCA1 has been reported to up-regulate the activity of the base excision repair (BER) pathway through a transcriptional mechanism that involves stimulation of the expression of several key BER enzymes (OGG1, NTH1, and REF1/APE1) (Saha et al., 2010a,b). BER is the major pathway for the repair of oxidized DNA and is normally an error-free process. Failure to repair different types of oxidized DNA lesions can result in cytotoxicity or mutagenesis, which can ultimately lead to cancer. The mechanism for up-regulation of BER enzyme expression was identified as stimulation of the activity of the OCT1. Previously, it was shown that BRCA1, like ATM, mediates a cytoprotective antioxidant response, characterized by stimulation of the activity of the antioxidant response transcription factor NFE2L2 (NRF2) (Bae et al., 2004). Further studies have revealed that BRCA1, in collaboration with REF1, down-regulates intracellular levels of reactive oxygen species (ROS), oxidized DNA, and nitrated proteins (Saha et al., 2009). However, the contribution of these functions of BRCA1 to tumor suppression is unknown.

The Fanconi anemia network consists of a group of proteins involved in the repair of DNA interstrand cross-links (ICLs), mutations of which lead to Fanconi anemia, a genetic disorder characterized by short stature, chromosomal instability, bone marrow failure, and increased sensitivity to agents that cause cross-linking of DNA. The accurate repair of ICLs involves, in part, HDR as well as the nucleotide excision repair (NER) pathway. Several studies suggest that BRCA1 participates in the repair of ICLs. These studies suggest two distinct roles for BRCA1 in ICL repair, one involving its function in HDR and the other independent of HDR (Zhou et al., 2005; Cheng et al., 2006; Bunting et al., 2012). However, the precise molecular functions of BRCA1 in the repair of ICLs are unclear. Conversely, as described above, several components within the Fanconi anemia network, interact with BRCA1 (directly or indirectly) and participate in BRCA1-dependent DNA repair of DSBs, including FANCI (=BACH1), FANCN (=PALB2), and FANCD1 (BRCA2).

In addition to ATM, BRCA1 is also phosphorylated at several sites by ATR in response to ultraviolet (UV) radiation (Tibbets et al., 2000; Gatei et al., 2001). These findings suggest a role for BRCA1 in the repair of UV damage, which is mediated, in part, by the NER pathway. In a recent study, it was reported that BRCA1 participates in the response to UV damage in a manner that is independent of the NER pathway (Pathania et al., 2011). Here it was found that following UV damage, BRCA1 is recruited through its BRCT domains to stalled replication forks, where

it participates in several processes including excision of photo-products and recruitment of components of the replication factor C (RFC) complex, with subsequent checkpoint activation and post-replicative DNA repair. Unlike BRCA1 recruitment to IRIF (which is delayed for more than 1 h), BRCA1 is recruited relatively rapidly (15 min) to sites of UV damage, primarily in S-phase cells (Pathania et al., 2011; Zhang et al., 2013). In one study, it was reported that BRG1, a component of the SWI/SNF chromatin remodeling complex, is required for BRCA1 recruitment to UV damage sites and that BRG1 modulates BRCA1 function in repair of UV damage by regulating the activation of ATR and ATM. Several other studies suggest roles for BRCA1 in the repair of UV damage (Navaraj et al., 2005; Marteijn et al., 2009) and it has been proposed that BRCA1 transcriptionally up-regulates genes involved in NER (Hartman and Ford, 2002).

BRCA1 AND PARP

Poly(ADP-ribose) polymerase (PARP) is a nuclear enzyme in the BER pathway that participates in the repair of single-strand breaks (SSBs) of DNA. Using a “synthetic lethal” screen, it was found that inhibition of PARP activity causes chromosomal instability and apoptosis in *BRCA1* or *BRCA2*-mutant cells but not in *BRCA1/BRCA2*-competent cells (Farmer et al., 2005). It was hypothesized that inhibition of PARP causes the accumulation of unrepaired SSBs that are then converted DSBs that would normally be repaired by HDR. This observation has led to clinical trials of small molecule PARP inhibitors (which had been originally developed as chemosensitizers) as a treatment for tumors arising in *BRCA1* and *BRCA2* mutation carriers (Lord and Ashworth, 2008). In a phase I trial of the PARP inhibitor olaparib (formerly AZD2281), significant responses were observed only in *BRCA1*-mutant and *BRCA2*-mutant cancers, with no responses in tumors wild-type for *BRCA* (Fong et al., 2009). In another phase I trial, 40% of patients with ovarian cancers due to germ-line *BRCA1/2* mutations achieved complete or partial responses with olaparib, with the response rates higher in *cis*-platinum sensitive tumors than in *cis*-platinum resistant tumors (Fong et al., 2010). Currently, there are eight PARP inhibitors under clinical investigation (with more under development) either as monotherapy, in combination with chemotherapy and radiotherapy, or in combination with other specifically targeted agents, for various types of malignancies (Papeo et al., 2013).

It should be noted that although PARP inhibitors still hold great promise in cancer therapy, *de novo* resistance or the development of resistance after an initial response has become problematic (reviewed in Montoni et al., 2013). For example, in a recent phase II trial, none of 26 patients with advanced triple negative breast cancer (tumors that lack estrogen and progesterone receptor expression and do not exhibit amplification of the HER2/Neu oncogene) had objective responses to the PARP inhibitor olaparib (Gelmon et al., 2011). In contrast a response rate of 41% was observed in ovarian cancer patients who carry *BRCA1* or *BRCA2* mutations (Gelmon et al., 2011). One very interesting study of tumor tissue derived from patients with *BRCA2* mutations that had initially responded to olaparib but subsequently developed resistance revealed secondary mutations in the resistant tumors that restored *BRCA2* function (Barber et al.,

2013). In a mouse model with a knock-in cancer-associated *Brcal* mutation-Cys61Gly), the tumors rapidly developed resistance to both olaparib and *cis*-platinum but retained the *Brcal* mutation.

CONCLUSIONS AND PERSPECTIVES

Breast and ovarian cancer susceptibility gene 1 is a tumor suppressor gene, inherited mutations of which confer a significantly increased risk breast and ovarian cancers. BRCA1 functions in the error-free repair of DSBs of DNA by HDR (also known as homologous recombination). This function appears to be critical for its tumor suppressor activity. BRCA1 may also function in a subtype of non-homologous end joining that is more accurate than classical error-prone NHEJ, although the role of BRCA1 in NHEJ is not settled. BRCA1 participates in a number of DNA damage-activated cell cycle checkpoints (e.g., intra-S and G2/M checkpoints) and in the response to stalled replication forks (e.g., those caused by DNA cross-linking agents). BRCA1 in complex with BARD1 exerts an E3 ubiquitin ligase activity that was once thought to be essential for tumor suppression, but this view was contradicted by a recent study of a transgenic mouse model homozygous for an engineered mutant *Brcal* gene that is defective for ubiquitin ligase activity but retains the ability to mediate HDR, since these mice did not develop cancer.

Since BRCA1 expression is widespread, the function of BRCA1 in mediating HDR and other DNA repair processes does not by itself explain the predilection of *BRCA1*-mutation carriers to develop such a limited range of tumor types, mostly breast and ovarian cancers. It is well-established that breast cancer is an estrogen-driven tumor type. Thus, the ability of BRCA1 to inhibit estrogen receptor activity (described above) could contribute to breast cancer suppression. Here, the idea is that during tumor development, mammary epithelial cells that exhibit genomic instability are stimulated to proliferate excessively because they lack a major mechanism that limits estrogen-stimulated proliferation. This hypothesis is consistent with findings suggesting that *BRCA1*-related tumorigenesis is hormonally responsive, at least in the early stages. It is expected that BRCA1 also mediates an ovary-specific function that could explain why the ovary is a preferred site for cancer development in women who carry *BRCA1* mutations.

It was also proposed that BRCA1 functions, including tumor suppression, can be explained by the ability of BRCA1 to ubiquitinate the histone H2A within satellite DNA, thus maintaining heterochromatin in a transcriptionally silenced state. Moreover, a clinical-epidemiologic study suggests that mutations mapping to the C-terminal region of BRCA1, which would be predicted to disrupt BRCA1 function in HDR, do not abrogate the ability of BRCA1 to suppress ovarian cancer. Thus the mechanisms by which BRCA1 suppresses breast and ovarian cancer development may differ. It is worthwhile to note that while mouse models of *Brcal*-dependent mammary tumorigenesis yield tumors with many of the characteristics of the human cancers, these models do not fully mimic the human situation. In addition to the obvious differences between mice and humans, *BRCA1*-related tumorigenesis in mouse and humans differ in other characteristics. Thus, in mice, a homozygous *Brcal*-mutation is targeted to the mammary gland or other organs, often along with a heterozygous or homozygous deletion of p53. On the other hand, humans inherit

one mutant *BRCA1* allele, with the other allele being wild-type. And since the mutation is in the germ-line, all somatic cells are heterozygous for *BRCA1*. Thus, the pathway for *BRCA1*-related tumorigenesis may not be the same in humans and mice.

We have reviewed evidence that *BRCA1* serves other DNA damage-related functions, including the regulation of telomere length and stability. Consistent with other DDR proteins that have complex roles in telomere biology (e.g., MRN, ATM, DNA-PK, and others), *BRCA1* exerts multiple telomere-related functions. Thus, *BRCA1* inhibits telomerase activity and causes telomere shortening, while at the same time preserving telomere stability by increasing the length of the G-strand overhang. As a result, extremely short telomeres in *BRCA1* over-expressing tumor cells did not trigger senescence, apoptosis, or cell cycle arrest. Although the telomerase activity is reduced in *BRCA1* over-expressing cells, it apparently remains sufficient to maintain the length of the shortened telomeres and synthesize the 3' G-strand overhang.

The mechanism by which *BRCA1* causes telomere shortening remains to be determined, since the rate of shortening was too

rapid to be due to the loss of telomerase activity alone. The complete absence of functional *BRCA1* creates telomere dysfunction, evidenced by the appearance of chromosomal aberrations of the type due to critical telomere shortening. However, assuming that *BRCA1* functions similarly in non-tumor cell types, the ability of *BRCA1* to inhibit telomerase activity and cause telomere shortening are consistent with a tumor suppressor function. To what extent these activities actually contribute to the tumor suppressor activity of *BRCA1* is unclear at present.

Finally, while much progress has been made in understanding how *BRCA1* is recruited to IRIF and its function during the DDR, its precise molecular functions in HDR and other DNA repair activities (e.g., NEHJ and ICL cross-link repair) remain to be determined. Furthermore, in understanding the role of HDR in *BRCA1*-mediated tumor suppression, it should be realized that mutations that disrupt this function are likely to disrupt many other functions of *BRCA1*; and at present, it is unclear which of these other functions contribute to tumor suppression and to what extent.

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The dual nature of mismatch repair as antimutator and mutator: for better or for worse

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DNA is constantly under attack by a number of both exogenous and endogenous agents that challenge its integrity. Among the mechanisms that have evolved to counteract this deleterious action, mismatch repair (MMR) has specialized in removing DNA biosynthetic errors that occur when replicating the genome. Malfunction or inactivation of this system results in an increase in spontaneous mutability and a strong predisposition to tumor development. Besides this key corrective role, MMR proteins are involved in other pathways of DNA metabolism such as mitotic and meiotic recombination and processing of oxidative damage. Surprisingly, MMR is also required for certain mutagenic processes. The mutagenic MMR has beneficial consequences contributing to the generation of a vast repertoire of antibodies through class switch recombination and somatic hypermutation processes. However, this non-canonical mutagenic MMR also has detrimental effects; it promotes repeat expansions associated with neuromuscular and neurodegenerative diseases and may contribute to cancer/disease-related aberrant mutations and translocations. The reaction responsible for replication error correction has been the most thoroughly studied and it is the subject to numerous reviews. This review describes briefly the biochemistry of MMR and focuses primarily on the non-canonical MMR activities described in mammals as well as emerging research implicating interplay of MMR and chromatin.

Keywords: non-canonical mismatch repair, antibody diversification, class switch recombination, somatic hypermutation, neurodegenerative diseases, trinucleotide repeats, chromatin modifiers

INTRODUCTION

The mismatch repair (MMR) system provides two main genetic stabilization functions; it is involved in the correction of errors generated during replication that escape polymerase proofreading and ensures the fidelity of recombination. Such a corrective role was first proposed to explain gene conversion in fungi (Holliday, 1974). Studies using bacteria and yeast uncovered MMR as a long patch correction system and identified its protein components (Grilley et al., 1990). The MMR process was then reconstituted using bacterial (Lahue et al., 1989), yeast (Bowen et al., 2013), and mammalian proteins (Constantin et al., 2005; Zhang et al., 2005). Defects in this pathway were shown to give rise to a mutator phenotype in bacteria and yeast with characteristic traits at repetitive sequences of simple nature, microsatellites (microsatellite instability, MSI; Levinson and Gutman, 1987; Strand et al., 1993). The observation that a subset of colorectal tumors contain a large number of mutations in microsatellite sequences was subsequently explained by the finding that these tumors were defective in MMR (Fishel et al., 1993; Leach et al., 1993; Jiricny, 1994; Modrich and Lahue, 1996). The discovery that MMR defects predispose to cancer (Lynch syndrome) highlighted the relevance of MMR in human disease and renewed the interest in MMR proteins, their structure, mechanisms of action and gene variants that may contribute to the disease (Boland and Goel, 2010). The mechanistic insights obtained by these studies did advance our understanding on how hereditary sequence variants in the minimal human MMR system affect the MMR

function and hence predispose to the DNA instabilities linked to cancer predisposition. The list of cancer types where MMR malfunction has been observed expanded to include the most frequent hereditary predisposition to colorectal cancer along with increased risk for development of endometrial, ovarian, gastric, small bowel, urothelial, brain, hepatobiliary, pancreatic, bladder, kidney, prostate and breast cancers, and hematological malignancies (Scott et al., 2001; Umar et al., 2004; Grindeland et al., 2009; van Oers et al., 2010; Wimmer and Kratz, 2010; Buerki et al., 2012; Win et al., 2012a,b; Vasen et al., 2013). The ability to predict cancer predisposition by analyzing the sequence variants for the MMR genes also contributed to better management of patients and their relatives and resulted in reduced mortality (Järvinen et al., 2009). Therefore, the characterization of such gene variants has become of prime interest and is nowadays a multidisciplinary international endeavor (Thompson et al., 2014). The efforts made in understanding MMR mechanism and function also led to the discovery of new roles for MMR. MMR was found to be involved in DNA damage signaling and intriguingly also in mutagenic processes such as somatic hypermutation (SHM), class switch recombination (CSR), and instability of trinucleotide repeats (TNRs; Hsieh, 2001; Li, 2008; Pena-Diaz and Jiricny, 2012; Edelbrock et al., 2013; Jiricny, 2013). This review describes first the components of mammalian MMR and their mode of action and then focuses on DNA transactions in which MMR contradicts its role as antimutator to become a mutator.

THE BIOCHEMISTRY OF MAMMALIAN MMR

Replication errors represent a considerable threat to genomic integrity. Failure to repair base-base mismatches and insertion/deletion loops (IDLs) arising during DNA replication increases mutation frequencies by two to three orders of magnitude. MMR associates with replication factories (Hombauer et al., 2011; Lopez-Contreras et al., 2013; Sirbu et al., 2013) and targets the newly synthesized DNA strand for repair thereby contributing to the fidelity of replication. MMR achieves this feat by a sequential mechanism comprising mismatch recognition, excision, and resynthesis steps. This process has been described in detail in several reviews (Kunkel and Erie, 2005; Jiricny, 2006; Modrich, 2006; Hsieh and Yamane, 2008). Briefly, the reaction commences by the binding of the MutS heterodimer to a mismatch (**Figure 1**). The MutS heterodimer is formed by either MSH2/MSH6 (MutS α) or MSH2/MSH3 (MutS β). Two other homologs, MSH4 and MSH5, have specific roles in meiosis and have been discussed previously (Snowden et al., 2004; Her et al., 2007). The MutS α complex recognizes single base mismatches and 1–2 nucleotide IDLs, while the MutS β complex recognizes

larger loops. The mechanisms of lesion recognition by MutS α and MutS β differ but in both cases binding leads to bending of DNA (Warren et al., 2007; Gupta et al., 2012). MutS heterodimers belong to the ABC transporter superfamily and contain ATP binding domains essential for MMR. Following substrate recognition, MutS undergoes an ADP–ATP exchange-driven conformational change into a sliding clamp and recruits the MutL heterodimer. There are several MutL homologs; MutL α , MutL β , and MutL γ that belong to the GHKL ATPase family (Dutta and Inouye, 2000). MutL α (MLH1/PMS2 heterodimer) is the prevalent homolog in MMR. MutL β (MLH1/PMS1) appears to lack a function in MMR, whereas MutL γ (MLH1/MLH3) contributes to some extent to MMR *in vitro* (Cannavo et al., 2005) but is primarily involved in meiotic recombination (Lipkin et al., 2002). The complex formed by MutS–MutL can translocate in either direction along the DNA contour in search of a strand discontinuity. When it encounters a strand discontinuity (such as a gap between Okazaki fragments) bound by PCNA, loading of the exonuclease EXO1 initiates degradation of the nicked strand that will terminate past the mismatch. Additionally, the latent endonuclease activity

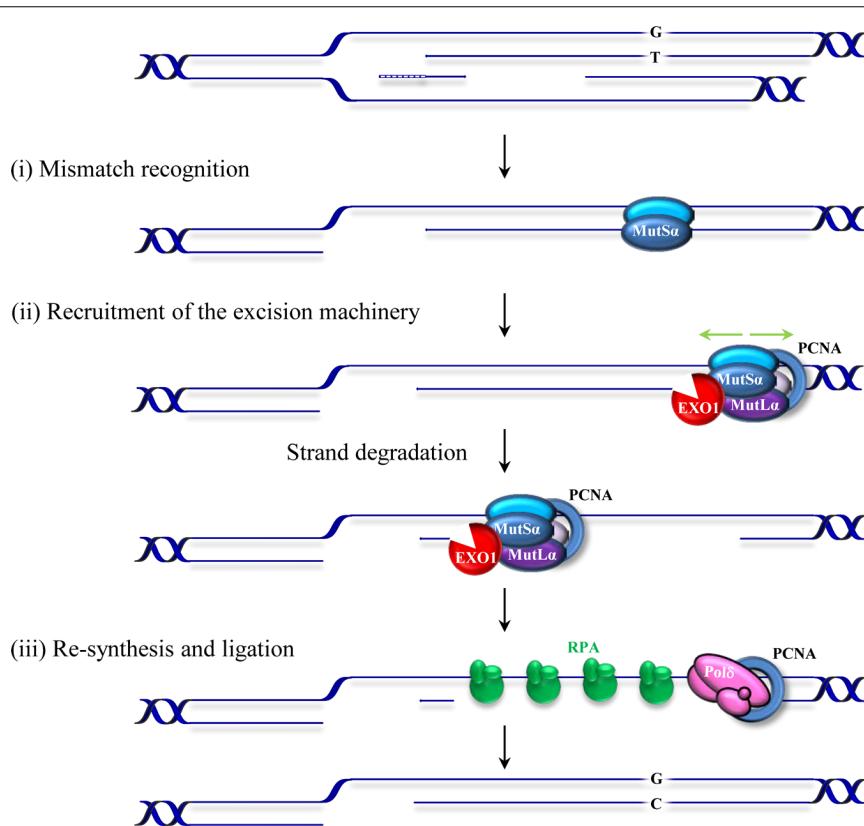


FIGURE 1 | Schematic representation of postreplicative mismatch repair in human cells. The canonical MMR process commences by the binding of the MSH2/MSH6 heterodimer, MutS α , to a mismatch (in this figure a G/T mismatch in the leading strand resulting from misincorporation during replication of thymidine opposite to guanosine). Upon binding, MutS α undergoes an ATP-driven conformational change and recruits the MLH1/PMS2 heterodimer (MutL α). This complex can translocate in either direction along the DNA contour (green arrows).

When it encounters a strand discontinuity (such as a gap between Okazaki fragments in the lagging strand or a PMS2 induced nick in the leading strand, not shown) PCNA binding (blue circle) and loading of an exonuclease (EXO1) initiate degradation of the nicked strand that will terminate past the mismatch. The resulting RPA-stabilized single-stranded gap is then filled in by the replicative polymerase and the remaining nick sealed by DNA ligase I. Small insertion/deletion loops (not shown) are corrected in a similar fashion by a MutS β (MSH2/MSH3) initiated process.

harbored by MutL α (Kadyrov et al., 2006) may provide an entry site for EXO1-dependent excision or for polymerase-dependent strand displacement reactions (Kadyrov et al., 2009). The resulting single-stranded gap is stabilized by RPA and then filled in by polymerase δ . The remaining nick is sealed by DNA ligase I. The physical interactions of MutS and MutL with the replication factor PCNA and the constitutive presence of the MMR machinery at replication factories support the role of MMR as a postreplicative repair mechanism. However, several studies indicate that MMR proteins may also function outside of S-phase (Brooks et al., 1996; Zlatanou et al., 2011; Pena-Diaz et al., 2012). In contrast to the classical MMR activity described above, some of the activities derived from this replication-uncoupled MMR are mutagenic. Such a mutagenic non-canonical MMR (ncMMR) has been found to influence immunoglobulin diversification and the stability of TNRs.

MMR IN IMMUNOGLOBULIN DIVERSIFICATION

GENERATION OF ANTIBODY DIVERSIFICATION IN HUMANS

Our immune system is able to generate a staggering repertoire of antibodies in order to deal with the variety of antigens that we may encounter during our life time. The information required to synthesize this large number of antibodies is not directly contained in our limited genome. Instead, several mutagenic processes taking place at the immunoglobulin locus are responsible for altering the genetic information to create sufficient diversity. Antibody diversity is generated in a two-stage process. Early in B cell development, DNA breakage and rejoining events between variable (V), diversity (D) and joining (J) gene segments assemble immunoglobulin genes and allow the production of a primary repertoire of low affinity IgM antibodies (Jung et al., 2006; Schatz and Swanson, 2011). In mammals, a second diversification process that alters the sequence and structure at the immunoglobulin genes occurs after exposure of a B cell to an antigen. This secondary process entails SHM and CSR mechanisms and generates different classes of antibodies with higher affinities and specificities (Maizels, 2005; Di Noia and Neuberger, 2007; Teng and Papavasiliou, 2007; Peled et al., 2008; Stavnezer et al., 2008). SHM introduces mutations in the variable region of the Ig gene while CSR recombines the variable region to a downstream constant region in the Ig locus by a double-strand break (DSB) induced event. SHM and CSR are initiated by a shared event involving targeted DNA deamination catalyzed by the enzyme activation-induced deaminase (AID; Muramatsu et al., 1999, 2000; Bransteitter et al., 2003; Chaudhuri et al., 2003; Dickerson et al., 2003). The discovery of AID represented a milestone in the immunology field and initiated further studies into the molecular basis of SHM and CSR processes (Delker et al., 2009). AID converts cytosines to uracils in single-stranded DNA (Bransteitter et al., 2003; Chaudhuri et al., 2003; Dickerson et al., 2003; Figure 2A) and initiates mutagenic processes with the participation of low fidelity DNA polymerases and DNA repair pathways including base excision repair (BER), MMR, classical non-homologous end-joining and alternative end-joining. Ample genetic evidence has substantiated the seemingly paradoxical involvement of BER and MMR in this mutagenic process. Moreover, mutations in MMR proteins that affect different catalytic functions or physical interactions with other components of

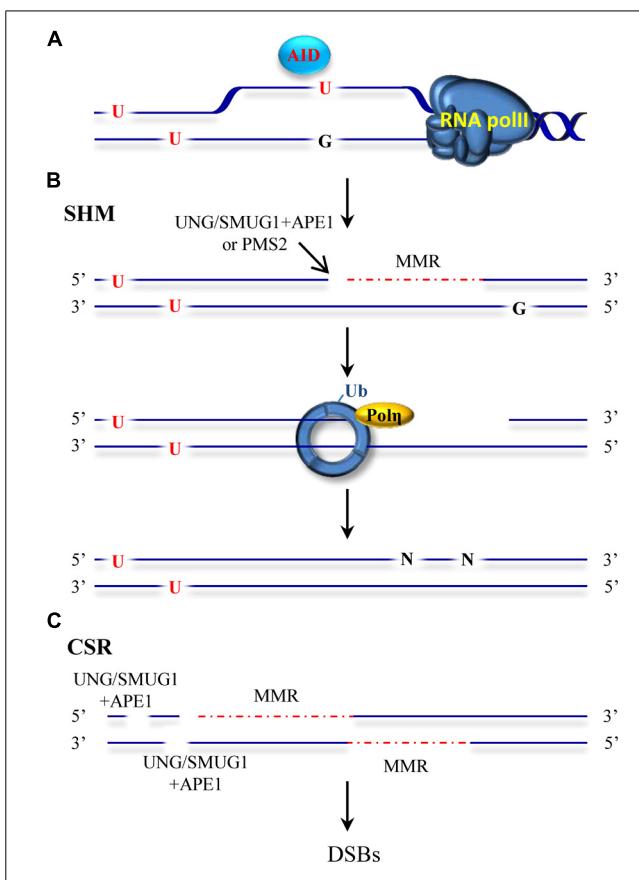


FIGURE 2 | ncMMR as a mediator in SHM and CSR. (A) AID deaminates cytosine to uracil in single-stranded DNA such as in DNA that is being transcribed. U:G mismatches can be recognized by the BER and MMR machineries. **(B)** An inefficient BER can lead to excision of the uracil by UNG or SMUG1 glycosylases and to an incision by APE1. MMR loaded at a different mismatch, can use this APE incision as an entry point for EXO1-mediated degradation. Alternatively, PMS2 endonuclease can generate the required entry site. The single-stranded DNA generated by EXO1 is not readily filled and promotes PCNA-Ub and recruitment of Pol η . Resynthesis by the error prone Pol η leads to mutations at different sites than the original deaminated cytosine. **(C)** Incisions generated by BER and/or MMR-dependent strand degradation can lead to DSBs when the degradation tracks and breaks are in close proximity on opposite strands. DSBs induction initiates recombination events during CSR. The red dashed line indicates MMR-dependent strand degradation.

this pathway have been shown to affect immunoglobulin diversification processes (Chahwan et al., 2011). This review summarizes the current mechanistic model proposed for mutagenic MMR.

MMR AS A MUTATOR AT THE IMMUNOGLOBULIN LOCUS

How is ncMMR engaged at the immunoglobulin locus? AID-mediated cytosine deamination results in a U/G mismatch in the DNA that leads to several outcomes. During SHM, if the mismatch is not corrected, replication across U will lead to C/G to T/A transitions. A second type of mutations is dependent on error-prone BER. BER initiated predominantly by the uracil–DNA glycosylase UNG, or to a minor extent by the backup activity of SMUG1 (Dingler et al., 2014) may correct the mismatch and restore the original sequence or, when incomplete, leave abasic sites that are

mis-repaired by error-prone polymerases. These events take place at the deaminated cytosine site and leads to both transitions and transversions (Sousa et al., 2007). These activities were confirmed by the finding that ablation of UNG in mice leads to accumulation of uracil in the DNA of immunoglobulin genes, and to a significant increase in transition mutations at C/G pairs (Rada et al., 2002; Maul et al., 2011). A third type of frequent mutations occurring at A/T base pairs and therefore not affected directly by the deamination of cytosine were shown to arise by a different mechanism (**Figure 2B**). This third mode of addressing uracil in DNA required long path DNA repair processes with a propensity to introduce errors. Genetic evidence suggested the involvement of MMR proteins, EXO1, mono-ubiquitylation of PCNA (PCNA-Ub) and primarily the translesion synthesis polymerase η (Bardwell et al., 2004; Delbos et al., 2007; Krijger et al., 2009; Chahwan et al., 2012a; Saribasak and Gearhart, 2012). Upon MutS α recognition of the U/G mismatch the complex slides along DNA in search of an entry site for EXO1 loading, and once such an entry site is found, initiates strand degradation. The gaps formed in this process are believed to persist and to trigger PCNA-Ub and recruitment of pol η . In absence of MSH2, mutations at A:T sites are drastically reduced but not completely abolished. In this scenario, BER is suggested to provide a backup role for the recruitment of pol η during SHM (Delbos et al., 2007). Two major open questions about this process remain: (i) which enzymatic activity generates the entry site for EXO1-dependent degradation and (ii) what distinguishes high fidelity from error prone MMR. (i) The answer to the first question may lie in the potential of AID to create clustered mutations at the Ig locus (Storb et al., 2009). In this scenario, BER may introduce a strand discontinuity that can be used by proximally loaded MMR proteins as entry point for EXO1 (Schanz et al., 2009). A complementary model substantiated by recent findings suggests that in absence of proximal entry sites, a cryptic endonuclease activity harbored by PMS2 may serve as a back-up for the DNA incision required to initiate EXO1-dependent strand degradation (Pluciennik et al., 2010; Pena-Diaz et al., 2012; Zivojnovic et al., 2014). Overlapping roles of BER and ncMMR have been demonstrated and endorse these two possibilities (Rada et al., 2004; Shen et al., 2006). Whereas strand discontinuities created by BER may serve to direct MMR to the same strand containing the nick, in absence of entry sites, the back-up cleavage by PMS2 endonuclease is largely without strand bias (Pluciennik et al., 2010; Pena-Diaz et al., 2012). The interplay between BER and MMR thus may affect the strand bias observed for mutations at A/T sites (preferential targeting of A nucleotides for mutation within WA motifs on the non-transcribed strand). The source of the strand bias observed at A/T sites though remain controversial (Franklin and Blanden, 2008; Frieder et al., 2009; Steele, 2009; Roa et al., 2010). (ii) Once EXO1 is loaded and strand degradation takes place, what distinguishes high-fidelity from error-prone MMR? Whereas high-fidelity MMR is coupled to replication, ncMMR acting in SHM and CSR processes may take place outside of S-phase. The mutagenic ncMMR thus may function in an environment where replicative polymerases are scarce and dNTP pools suboptimal. This could lead to inefficient refilling of the single-stranded gaps formed during the repair process, which would in turn elicit PCNA-Ub and

promote refilling of the gap by error-prone polymerases. In this model, DNA lesions addressed by MMR outside S-phase promote MMR-dependent PCNA-Ub. This is supported by several studies showing that oxidative and alkylating DNA damage can elicit MMR-dependent PCNA-Ub independently of the cell-cycle phase (Schroering and Williams, 2008; Zlatanou et al., 2011; Pena-Diaz et al., 2012).

CSR similarly to SHM requires AID, BER, and MMR proteins. CSR requires the formation of DSBs in highly repetitive switch regions located upstream of each of the heavy chain constant region genes (**Figure 2C**). These breaks are subsequently processed by canonical non-homologous end-joining (C-NHEJ) that seals DNA ends with little or no homology or by alternative end-joining (A-EJ) that requires microhomology for ligation (Boboila et al., 2012; Cortizas et al., 2013). How these DSBs are created is not entirely clear. BER may create single strand-breaks on opposite strands that when sufficiently close lead to DSBs (Masani et al., 2013). Fortuitous overlap of MMR-generated gaps with BER breaks or other MMR-induced gaps in the opposite strand provides an additional explanation for the formation of DSBs (Peron et al., 2008; van Oers et al., 2010). Strikingly, while SHM is largely independent of MutL α , the formation of DSBs during CSR requires the PMS2 endonuclease activity (van Oers et al., 2010). MMR can be initiated using strand discontinuities provided by BER and therefore does not strictly require PMS2 endonuclease activity (Genschel and Modrich, 2003). In this scenario, the gaps formed by MMR are in the same strand than the original strand discontinuity provided by BER. On the other hand, formation of gaps on the opposite strand of nicks generated by BER is aided by the lack of strand bias exhibited by MMR in absence of nearby nicks (Pluciennik et al., 2010; Pena-Diaz et al., 2012). This therefore increases the likelihood of DSB formation and it may partly explain the critical requirement of PMS2 endonuclease activity during CSR. MMR proteins may have additional functions beyond their major role converting AID DNA damage into suitable broken ends for C-NHEJ and A-EJ pathways. Recent studies suggest that MMR proteins may influence the pathway choice for resolution of the DSBs formed during CSR (Eccleston et al., 2011; Chahwan et al., 2012b; Cortizas et al., 2013). Biochemical evidence substantiating the models for DSBs formation during CSR and the potential role of MMR proteins in pathway choice for DSBs resolution is still missing.

Currently, it is not known whether the ncMMR mutagenic activity is engaged exclusively at AID deaminated sites in the immunoglobulin locus. AID may act on many non-Ig genes (Liu et al., 2008; Chiarle et al., 2011; Klein et al., 2011; Staszewski et al., 2011; Fear, 2013) and spontaneous deamination of cytosine to uracil is also a frequent event (~200 per mammalian genome per day; Kavli et al., 2007). Therefore, lesions that can be recognized by MMR are not locus specific and MMR mutagenic activities may be more frequent than anticipated. The interplay or competition between BER and MMR activities, the regulation of the access of error-prone polymerases and the timing of repair related to the cell cycle are likely to influence the balance between high-fidelity and error-prone DNA repair in these loci.

MMR IN NEURODEGENERATIVE DISEASE

REPEAT INSTABILITY AS A CAUSE OF HUMAN DISEASE

Expansion of simple repeats in genomic DNA is the underlying cause of over 30 human neurodegenerative and neuromuscular inherited diseases such as Huntington's disease (HD), myotonic dystrophy type 1 (DM1), fragile X syndrome type A (FRAXA), Friedreich's ataxia (FRDA), and spinocerebellar ataxias (SCAs). Unstable repeats are polymorphic and show a normal range in healthy individuals, and a pathological range, i.e., above a threshold length, associated with clinical manifestations. Instability can occur during both meiotic and mitotic divisions and at various stages of the cell cycle (Nag, 2003; McMurray, 2010). Several of the repeat expansion-associated diseases show anticipation, in which subsequent generations display earlier disease onsets. Otherwise, somatic instability accounts for increases with age towards larger size of the repeats in a tissue-dependent manner correlating with progression of the symptoms. Long repeats exceeding a determined threshold tend to be more unstable and both gametic and mitotic instability becomes more likely with increasing repeat length. The unstable repeats can be found at different regions of their resident genes (Mirkin, 2007) and the etiology of the diseases caused by their expansion reflects this diversity. Repeat expansions can cause disease by a variety of both loss and gain of function pathways, interfering with the expression or properties of the gene products, affecting splicing or antisense regulation. The most common unstable disease-associated DNA repeats are TNRs including CAG, CTG, CGG, and GAA triplets and their expansion is thought to be linked to their ability to form unusual secondary structures (Gacy et al., 1995; Mirkin, 2007). Several mechanisms including errors during DNA replication, meiotic recombination, transcription, DNA repair, and chromatin remodeling have been proposed to contribute to the observed instability (Lin et al., 2009; Lopez Castel et al., 2010; McMurray, 2010; Kim and Mirkin, 2013), but their relative contribution remains unknown.

MMR AS A SOURCE OF REPEAT INSTABILITY

The involvement of DNA repair mechanisms in repeat expansion was suggested to explain the puzzling finding that in diseases such as HD, somatic repeat instability appears most pronounced in non-proliferating tissues of the CNS (Gonitel et al., 2008) and that repeat expansion rates did not always correlate with cell division rates (Lia et al., 1998; Fortune et al., 2000; Gomes-Pereira et al., 2001). The first evidence that the MMR system contributes to repeat expansion was obtained by Manley et al. (1999). Given that a functional MMR is required for maintaining the stability of microsatellite sequences (mostly mono- and dinucleotide repeats) the authors set out to analyze whether MMR affects the stability of HD-associated CAG repeats. Surprisingly, *Msh2*^{-/-} transgenic mice bearing a copy of the human HD exon 1 (containing the CAG repeats), showed reduced expansion of the introduced (CAG)_n repeats when compared with *Msh2*^{+/+} HD exon 1 mice counterparts. Additional studies confirmed this novel mutagenic role of *Msh2* in HD CAG repeat instability and HD CAG-dependent phenotypes (Kovtun and McMurray, 2001; Wheeler et al., 2003; Kovalenko et al., 2012). However, the observation that *Msh2* deficiency did not completely abolish expansions suggested further

hitherto unknown roles for other DNA repair processes in promoting repeat instability. Later studies provided further evidence for the non-canonical role of *Msh2* in trinucleotide repeat instability, this time in (CTG)_n repeat expansion associated with DM1. In contrast to the observations in HD, *Msh2* absence resulted in a shift towards (CTG)_n contraction rather than stabilization of the repeat size (Savouret et al., 2003). These initial findings led to a number of studies designed to decipher the role of MMR in repeat expansion. The involvement of other components of the MMR machinery was subsequently analyzed. *Msh3* deficiency was found to block somatic (CTG)_n expansions in DM1 knock-in mice whereas *Msh6* deficiency increased the frequency of such events (Foiry et al., 2006). This suggested competition of *Msh3* and *Msh6* for binding to *Msh2* and differential effects of MutS α and MutS β complexes in repeat expansion (van den Broek et al., 2002). Wheeler and coworkers confirmed separate functional roles of MutS α and MutS β complexes in HD knock-in mice and showed that whereas *Msh6* protects against intergenerational contractions, *Msh3* is required for CAG expansions in striatum (Dragileva et al., 2009). A model to account for the role of MutS β in repeat instability proposes that MutS β -dependent stabilization of secondary structures formed at the repeats and uncoupling from downstream repair events leads to instability (Figure 3A; McMurray, 2008). In addition, the requirement for the MutL α component PMS2 (Gomes-Pereira et al., 2004) suggested a second model where repeat instability requires a fully functional MMR (Figure 3B). This model is supported by the finding that *Msh2*-mutant mice carrying a missense mutation *Msh2*^{G674A/G674A} show less pronounced CTG expansions than wild type mice (Tome et al., 2009). This mutation retains mismatch recognition activity but fails to support MMR *in vitro* (Lin et al., 2004; Ollila et al., 2008; Geng et al., 2012). In an effort to gain a mechanistic insight into the MMR-dependent instability process, biochemical studies were undertaken. Using synthetic DNA substrates containing CAG or CTG slipped out structures a third model was suggested where MSH2, MSH3, and PMS2 mediate the formation of expansion intermediates prior to processing of the slip-outs (Figure 3C; Panigrahi et al., 2005). In this model, repair is triggered either by DNA damage in or near the TNR, or by the aberrant TNR-DNA structure itself. Subsequent excision of nucleotides is followed by error-prone repair synthesis. Despite this wealth of knowledge, the biochemical role of MutS β in repeat instability remains controversial. MutS β processing of CAG slip-outs *in vitro* may depend on assay conditions as well as the size, number and structure of the hairpins (Owen et al., 2005; Tian et al., 2009; Panigrahi et al., 2010; Lang et al., 2011; Zhang et al., 2012). The involvement of the MutL α heterodimer in repeat instability was also analyzed *in vitro*. Pearson and coworkers demonstrated that a functional MutL α complex is required for processing (CAG)_n or (CTG)_n extrusions (Panigrahi et al., 2012). How PCNA-dependent activation of MutL α endonuclease occurs in the context of non-replicating DNA was later revealed by the finding that repeat extrusions may serve as loading sites for the PCNA clamp (Pluciennik et al., 2013). These biochemical approaches have contributed to our understanding of MMR activities at unstable repeats. However, they yield only partial reactions at TNRs. Therefore, the combined use of the biochemical assays together with genetic (Dixon et al., 2004)

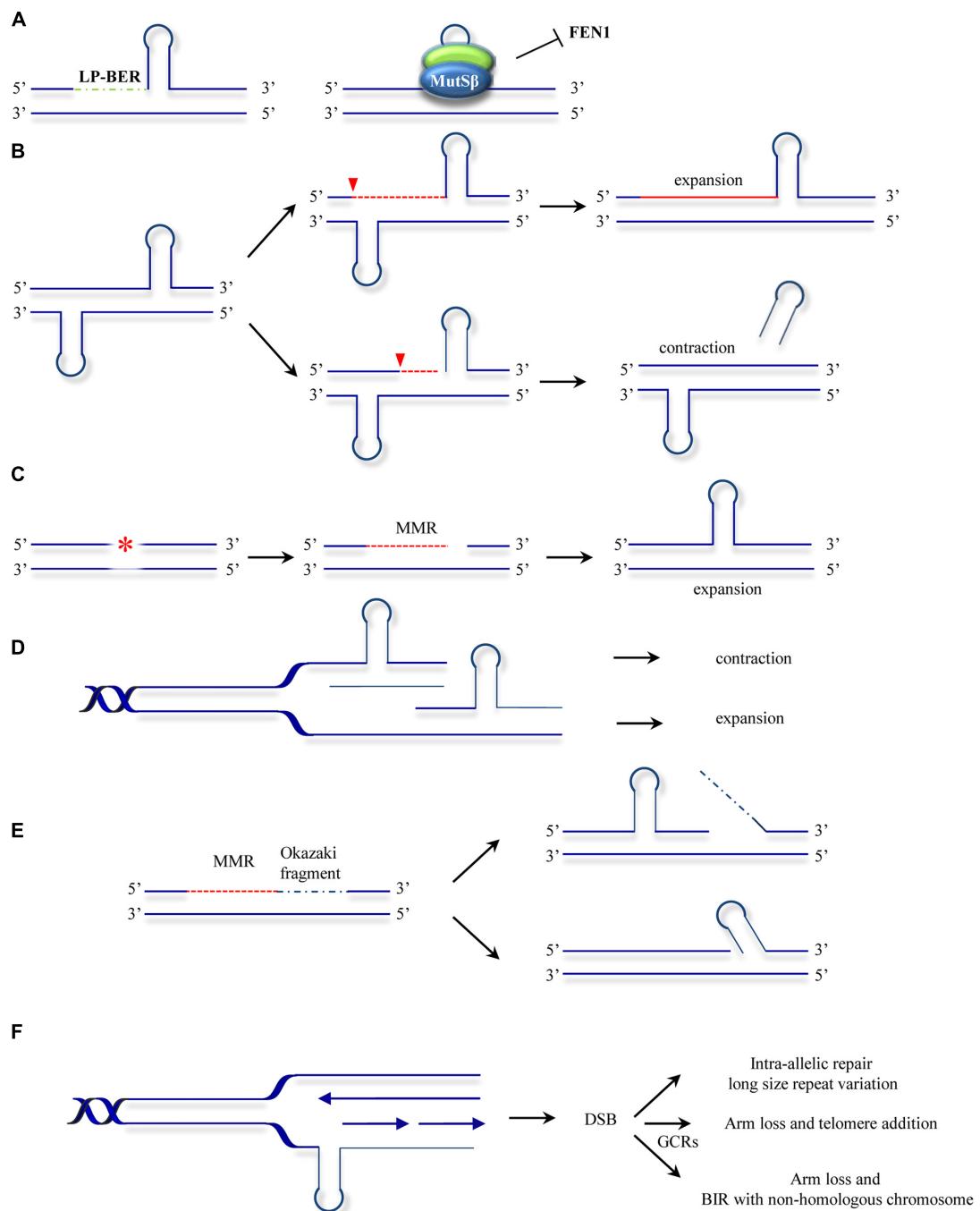


FIGURE 3 | Models for ncMMR function in repeat instability. (A) MMR hijacks and stabilizes the hairpins formed at repeats by strand displacement during long-patch BER (LP-BER). This inhibits further processing by other repair mechanisms such as FEN1 dependent flap removal (McMurray, 2008). **(B)** Nicking in the strand opposite to a hairpin leads to unwinding of the hairpin and resynthesis across resulting in repeat expansion. Processing of the strand containing the hairpin may lead to hairpin removal and repeats contraction (adapted from Gomes-Pereira et al., 2004). **(C)** Mismatch repair processing of lesions (e.g., oxidative or alkylating damage) may lead to strand degradation and faulty resynthesis resulting in hairpin formation. **(D)** As in the hijacking model **(A)**, MutS β stabilization of hairpins formed by polymerase slippage during replication or lack of processing of IDLs results in contraction

when the hairpin is located in the template strand or expansions when the hairpin is formed in the newly synthesized strand. **(E)** Gap filling can lead to hairpin formation by strand displacement during Okazaki fragment processing (Kantartzis et al., 2012; Kim and Mirkin, 2013). **(F)** Hairpins formed at the template strand can promote MMR processing leading to DSBs. The DSBs formed can be processed by different mechanisms leading to gross chromosomal rearrangements (GCRs) or to repeat length variations (Kim et al., 2008). The models presented here are not mutually exclusive. The asterisk represents a lesion addressed by MMR. The red dotted line indicates MMR-dependent processing including strand degradation and resynthesis steps. Inverted red triangles indicated the position of EXO1 entry site. BIR, break induced replication.

and *in vitro* assays where complete expansion can be recapitulated (Stevens et al., 2013) may contribute further to our knowledge about the mechanisms involved in MMR-mediated instability at TNRs.

The use of other model organisms may also shed some light on the MMR mutagenic activities. Instability of TNRs was also modeled in bacteria and yeast cellular systems. In contrast to the expansion bias observed in human neurodegenerative diseases, deletion events are more frequent in bacteria and yeast (Kovtun and McMurray, 2008). In these model organisms DNA replication seems to be the major contributor to repeat instability. Several replication models for repeat expansion have been drawn on the common basis that repetitive sequences posit a challenge for replication fork progression (Kim and Mirkin, 2013). Indeed, the earliest molecular model of how repeat expansions occur was based on DNA strand slippage during replication (Kunkel, 1993). In this first model, repeats misalign during replication, resulting in formation of extrahelical DNA loops. These loops may escape from correction or become stabilized by a MutS β -dependent mechanism. A subsequent round of replication will give rise to progeny DNA that is shorter than the template when the loop was located at the template strand or expanded when the misaligned nucleotides are in the newly synthesized strand (**Figure 3D**). Other studies suggest that MutS β interferes with normal processing of Okazaki fragments and promotes small expansion events (**Figure 3E**; Kantartzis et al., 2012). ncMMR has also been involved in expansions via replication fork stalling, DSB formation and repair (**Figure 3F**; Kim et al., 2008). These models also have support in mammalian cell systems, as repeats have been shown to interfere with replication (Follonier et al., 2013) and the direction of replication was found to influence the frequency of expansions (Claassen and Lahue, 2007). Investigations into SV40-driven replication of plasmid templates containing (CAG)n repeats in human cells also support a role for replication in promoting repeat instability (Panigrahi et al., 2002).

Another layer of complexity is added by the potential crosstalk between different DNA repair mechanisms in repeat instability. Formation and processing of secondary structures formed at repeats suggest cooperation between MMR and other DNA repair mechanisms such as BER (Kovtun and McMurray, 2007; Kovtun et al., 2007), NER (Lin and Wilson, 2009), and chromatin modifiers (Gannon et al., 2012). Interplay of MMR with BER and NER in other cellular processes has previously been suggested (Hong et al., 2008; Schanz et al., 2009; Zhao et al., 2009; Edelbrock et al., 2013) implying that such cooperation may be a conserved feature of DNA damage response mechanisms.

Given that several of the expandable repeats associated with disease can form unusual secondary structures, and that these structures are likely to be the underlying cause of instability, it is anticipated that ncMMR plays a role in TNRs-associated diseases other than HD and DM1. In fact, Msh2 was shown to reduce intergenerational expansion of (CGG)n in a FRAXA mouse model (Lokanga et al., 2013). Analyses of (GAA)n expansions associated with FRDA though led to conflicting results (Perdomini et al., 2013). The use of alternative models such as FRDA mouse models (Bourn et al., 2012; Ezzatizadeh et al., 2012),

FRDA induced pluripotent stem cells (Ku et al., 2010; Du et al., 2012) or ectopic expression of MSH2 and MSH3 in FRDA patient-derived fibroblasts (Halabi et al., 2012), may explain the discrepancies observed.

In addition, other MutS and MutL homologs may affect the stability of repeats. In this regard, a role for MutLy in TNR expansion associated with HD has recently been described (Pinto et al., 2013). Further work is needed to clarify the potential mutagenic role of ncMMR and the MMR proteins involved in these and other repeat-associated diseases.

The models described above are not mutually exclusive and reveal a high degree of unexpected context-dependency. The mechanisms of repeat expansion may differ depending on the sequence and length of repeat, replication rates, transcription rates, chromatin state, and crosstalk between different repair mechanisms. Future work is needed to understand the relative contribution of each of these mutagenic activities to the instability of repetitive sequences.

MMR IN THE CONTEXT OF CHROMATIN

Little is known about the influence of the chromatin context on MMR activity. Most reconstituted reactions used so far were minimal systems that cannot account for MMR as it may occur in the context of chromatin. Therefore, how the DNA packaging into chromatin affects MMR and how chromatin is restored after repair remains largely unknown. Nucleosomes inhibit MMR (Li et al., 2009) and MutS α diffusion (Gorman et al., 2010) and this barrier can be counteracted by MutS α -dependent nucleosome disassembly (Javaid et al., 2009). On the other hand, deposition of nucleosomes during replication may be tuned with MMR. By using *in vitro* modified systems containing chromatinized substrates, the groups of Jiricny and Kadyrov recently analyzed the mechanisms of nucleosome assembly during repair (Kadyrova et al., 2011; Schopf et al., 2012). These studies found coordination of MMR and nucleosome deposition initiated by the histone chaperone chromatin assembly factor 1 (CAF-1) and physical interaction between MutS α and CAF-1. CAF-1 is an essential factor in chromatin assembly in newly replicated DNA (Hoek and Stillman, 2003) and can function locally at NER sites (Green and Almouzni, 2003). The described crosstalk between MMR and CAF-1 is proposed to extend the time window available for repair by delaying chromatin assembly after replication. Histone modifications also contribute to the regulation of MMR in a chromatin context. The histone mark H3K36me3 was recently found to interact with the MMR protein MSH6 (Vermeulen et al., 2010) and facilitate MMR function by mediating its association with chromatin (Li et al., 2013). This mark is linked to actively transcribed regions but also peaks at the G1/S transition where it constitutes a chromatin signature for early replication domain boundaries (Ryba et al., 2010). This may contribute to explain the observed constitutive presence of MMR at replication factories (Lopez-Contreras et al., 2013; Sirbu et al., 2013) and its readiness for action. Importantly, mutations in SETD2, the histone methyltransferase responsible for H3K36 trimethylation, correlate with MSI found in renal cell carcinoma and Burkitt's lymphoma cell lines that do not display genetic or epigenetic defects in MMR genes. This may provide the molecular basis for MSI in cancer with otherwise intact MMR.

Similarly, histone H3 acetylation in yeast acts in concert with MMR in mutation avoidance (Kadyrova et al., 2013). These new findings pave the way for future research and a better understanding of the MMR role in disease.

CONCLUDING REMARKS

A common theme among the DNA sequences that are subjected to mutagenic repair seems to be their tendency to present an obstacle for transcription/replication machineries to proceed. So far, only few mutation-prone genomic loci have been described, but a large fraction of the genome contains sequences with these features. Thus, it is likely that mutagenic ncMMR is not restricted to the loci described but rather influences genome integrity to a larger extend. Comprehensive studies deciphering the global finger print of mutagenic ncMMR are then needed to understand how ncMMR affects genome maintenance and contributes to disease. In addition, future studies will have to determine the factors that direct the path choice towards mutagenic or corrective activities. In the past decades the finding that MMR is involved in Lynch syndrome highlighted the relevance of this DNA repair mechanism and led to a significant progress in the field. The novel findings on the role of ncMMR in mutagenic processes and the cross-talk of MMR with other DNA repair mechanisms and with chromatin architecture are likely to renew this interest. We are confident that deeper insight into mutator and anti-mutator activities of the MMR machinery will be the basis to develop novel improved strategies for the management and treatment of MMR-associated diseases.

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Alternative end-joining mechanisms: a historical perspective

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In the presence of functional DNA repair pathways, DNA double-strand breaks (DSBs) are mainly repaired by non-homologous end-joining (NHEJ) or homologous recombination (HR), two conserved pathways that protect cells from aberrant chromosomal rearrangements. During the past two decades however, unusual and presumably distinct DNA end-joining repair activities have been unraveled in NHEJ-deficient cells and these are likely to operate in various chromosomal contexts and species. Most alternative DNA end-joining events reported so far appear to involve microhomologous sequences and are likely to rely on a subset of HR enzymes, namely those responsible for the single-strand annealing mechanism of HR, and on DNA Ligase III. Usually, microhomologies are not initially present at DSB ends and thus need to be unmasked through DNA end resection, a process that can lead to extensive nucleotide loss and is therefore highly mutagenic. In addition to microhomology-mediated end-joining events, recent studies in mammalian cells point toward the existence of a distinct and still ill defined alternative end-joining pathway that does not appear to rely on pre-existing microhomologies and may possibly involve DNA Ligase I. Whether dependent on microhomologies or not, alternative DNA end-joining mechanisms are likely to be highly mutagenic *in vivo*, being able to drive telomere fusion events and cancer-associated chromosomal translocations in mouse models. In the future, it will be important to better characterize the genetic requirements of these mutagenic alternative mechanisms of DNA end-joining.

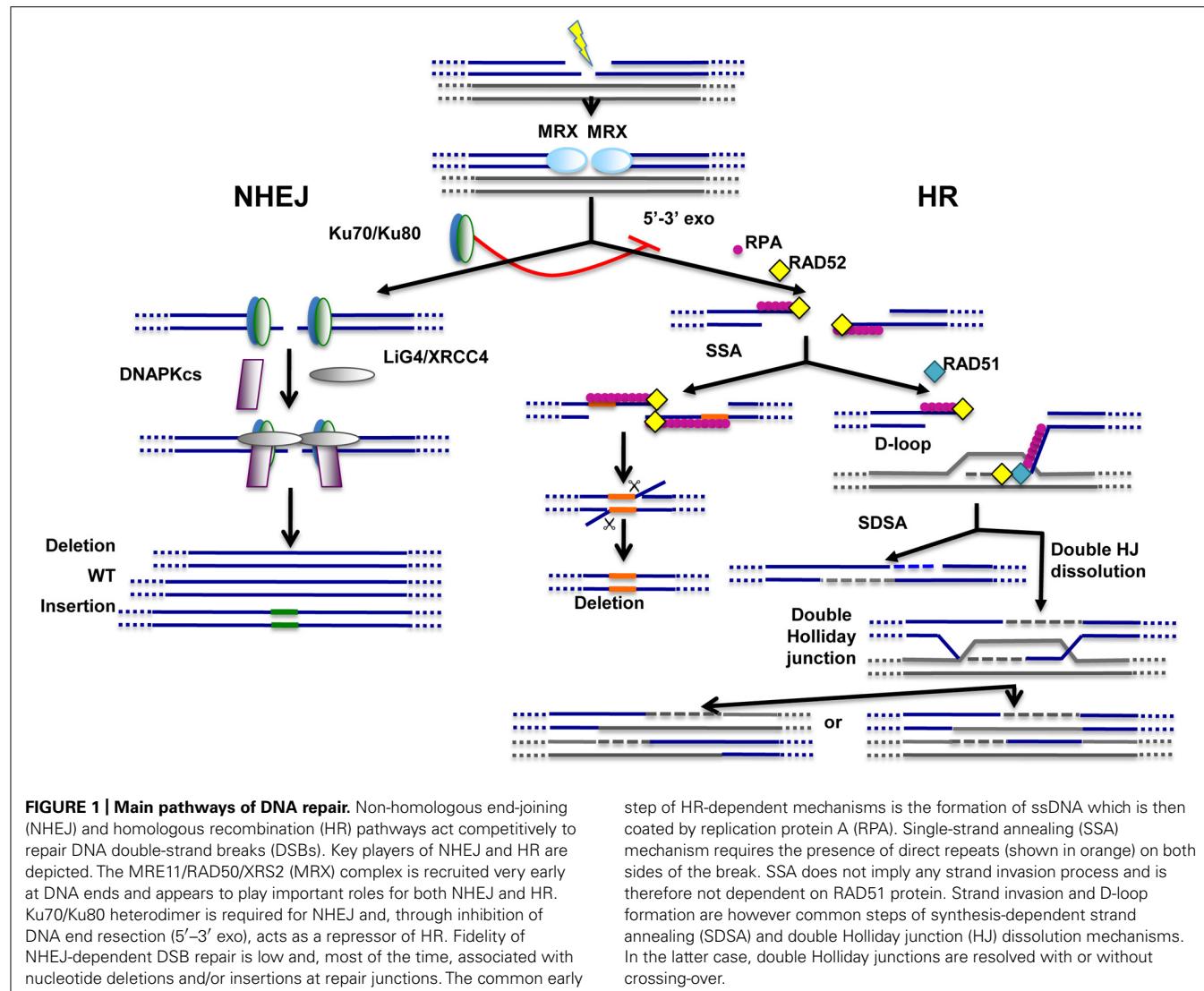
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INTRODUCTION

Double-strand breaks (DSBs) represent major threats to genome integrity. They can be induced during normal metabolism or may result from the presence of exogenous genotoxic agents like ionizing radiations or chemotherapeutic drugs. Cells have evolved two main pathways to repair these lesions: the non-homologous end-joining (NHEJ) pathway, that ensures direct resealing of DNA ends; and the homologous recombination (HR) pathway that relies on the presence of homologous DNA sequences for DSB repair. Repair through HR is not defined by a unique mechanism but operates through various mechanistically distinct DSB repair processes including synthesis-dependent strand annealing (SDSA), double Holliday junction resolution, and single-strand annealing (SSA; Paques and Haber, 1999; Chapman et al., 2012; **Figure 1**). The common step for HR-dependent DSB repair mechanisms is the initial formation of single-stranded DNA (ssDNA) for pairing with homologous DNA template sequences. These HR-dependent mechanisms of DSB repair have been extensively reviewed previously and will not be detailed here (Paques and Haber, 1999; Chapman et al., 2012).

Until very recently, how cells choose between NHEJ and HR-dependent pathways for DSB repair was still unclear, although both the cell cycle stage and the nature of DSB ends were previously involved (Symington and Gautier, 2011; Chapman et al.,

2012). A critical determinant for the choice is provided by the 5'-3' resection of DNA ends that, while triggering HR-dependent repair, prevents NHEJ. On the contrary, direct binding at DSB ends of the conserved Ku70/Ku80 heterodimer, a key complex of the NHEJ pathway that protects DNA ends against exonucleases, represses HR-dependent mechanisms (Pierce et al., 2001). Four studies recently shed new light on this important question of initial choice between NHEJ and HR for DSB repair and provided consistent evidences in favor of the existence of a tightly regulated mechanism (Chapman et al., 2013; Di Virgilio et al., 2013; Escribano-Diaz et al., 2013; Zimmermann et al., 2013). Four proteins were shown to play critical roles in repair pathway choice: RIF1 (Rap1-interacting factor 1), 53BP1 (p53 binding protein 1), BRCA1 (breast cancer type 1 susceptibility protein), and CtIP (C terminus-binding protein-interacting protein). Briefly, it appears that, while 53BP1–RIF1 stimulates NHEJ, BRCA1 and CtIP together promote DNA end resection and HR. RIF1 is indeed recruited to DSB ends via an interaction with 53BP1 and both proteins cooperate to promote NHEJ in G1 cells. During G2, RIF1/53BP1 binding to DNA ends is repressed by BRCA1, ensuring a switch to HR during this stage of the cell cycle. Collectively, these studies provide strong evidences in favor of well-regulated competitions between NHEJ and HR pathways at DNA ends.



The choice of DSB repair pathway inevitably impacts on the fidelity of repair. Indeed, while HR is generally viewed as a conservative DSB repair pathway, NHEJ operates with poor fidelity and nucleotide deletions and/or insertions are frequently detected at repair junctions (Figure 1). However, not all HR-dependent mechanisms display high fidelity of repair. Namely, the SSA mechanism requires annealing at two directly repeated DNA sequences near DSB ends. Consequently, intervening nucleotides initially present between the direct repeat sequences flanking the break are lost during SSA-mediated repair (Figure 1).

More recently, backup pathways for DNA repair were identified in NHEJ-deficient cells of various organisms. Although it is still unclear whether only one or several backup repair pathways exist, they do not rely on large homologous DNA templates such as those involved in HR-dependent repair events and are therefore referred to as “alternative end-joining” pathways. However, alternative end-joining mechanisms usually -but not always- rely on the presence of microhomologies at or near DSB ends,

suggesting that these repair events may not be entirely distinct from HR-dependent mechanisms. Here, I review the history of alternative end-joining discovery and the recent evidences that these alternative end-joining events are able to drive class switch recombination in the immune system, telomere fusions and chromosomal translocations *in vivo*.

ALTERNATIVE END-JOINING REPAIR: ONE OR MORE BACKUP PATHWAY(S)?

Proteins required for NHEJ include -but are not restricted to- the highly conserved Ku70/Ku80 heterodimeric complex, DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and DNA Ligase IV (LIG4) in complex with XRCC4 (Weterings and Chen, 2008). By directly binding DNA ends, Ku70/Ku80 ensures protection against exonucleases and, as such, acts as an inhibitor of HR (Figure 1). In 1996, thanks to the use of Ku70-deficient budding yeast mutants, Boulton and Jackson provided the first evidences for the existence of an alternative DNA end-joining pathway. This pathway was about 20-fold less efficient than

NHEJ and repair junctions displayed both nucleotide deletions and overlapping microhomologies of 3–16 nucleotides (Boulton and Jackson, 1996). Although it was known at that time that short microhomologous regions of up to five nucleotides were commonly recovered at NHEJ repair junctions of mammalian cells (Roth et al., 1985), this DNA repair pathway was clearly able to operate in a NHEJ-deficient background. Supporting the existence of a new DNA end-joining pathway, biochemical fractionation of calf thymus extracts yielded two fractions with distinct DSB repair activities (Mason et al., 1996). One fraction, presumably enriched for microhomology-mediated end-joining (MMEJ) activity, was clearly relying on the presence, on both sides of the DSB, of short repeat sequences. The second fraction, containing the NHEJ activity, was characterized by the presence of a DNA fill-in activity -inhibited by DNA polymerase inhibitors- and the ability to perform ligation of non-homologous DNA fragments (Mason et al., 1996). In agreement with the previous suggestion that very short sequence homologies are likely to help DNA end alignment prior to NHEJ-dependent repair (Roth et al., 1985; Roth and Wilson, 1986), some repair junctions produced by the NHEJ activity-containing fraction were also characterized by the presence of overlapping microhomologies. However, the remaining repair junctions were devoid of any microhomology, indicating that microhomologies were not strictly required for NHEJ in this system (Mason et al., 1996). Later on, backup pathways of end-joining were identified in various NHEJ-deficient mammalian cells (Kabotyanski et al., 1998; Feldmann et al., 2000; Wang et al., 2003).

Whether this newly identified backup MMEJ pathway was involving a new set of DNA repair proteins was unclear at that time. In 1994, and although they were working in a NHEJ-proficient budding yeast background, the group of Haber first postulated that microhomology-mediated DNA repair events may occur through a RAD52-dependent SSA-type mechanism (Kramer et al., 1994). The same group then reported that the lower limit for SSA-dependent DSB repair was lying between 5 and 29 bp of homology, showing that sequence homologies may be very low for HR, at least in budding yeast (Sugawara et al., 2000). They suggested however, that some differences may exist between classical SSA, involving large direct repeats, and “micro-SSA”, in which homology lengths are much lower, as the latter process appeared to rely mostly on RAD59, a budding yeast homolog of RAD52, instead of RAD52 itself (Sugawara et al., 2000). Altogether, studies by the group of Haber thus pointed toward the possible contribution of HR-dependent pathways in budding yeast MMEJ, suggesting that this may not represent a new completely distinct DNA repair pathway but could reflect a micro-SSA-type mechanism of DSB repair. In complete agreement with these predictions, a study performed in *X. laevis* eggs established that a purified fraction displaying MMEJ activity contained DNA Ligase III (LIG3), DNA polymerase ε, FEN-1 endonuclease, and exonuclease activities of 5'-3' and 3'-5' directionality and that the same fraction was able to process SSA intermediates (Gottlich et al., 1998). Next, it was reported that, in a NHEJ-deficient *S. cerevisiae* background, MMEJ events were not dependent on RAD52 but required the MRE11/RAD50/XRS2 complex previously implicated in both NHEJ and HR (Ma et al., 2003; Yu and Gabriel, 2003). The requirement for RAD59 was

however, not tested. Extrachromosomal DSB repair experiments in NHEJ-deficient fission yeast mutants then provided additional evidences in favor of a SSA-dependent mechanism for MMEJ (Decottignies, 2007). In this system, both RAD22, the fission yeast RAD52 homolog, and EXO1, the 5'-3' exonuclease involved in the formation of ssDNA intermediates for HR, were required for MMEJ (Decottignies, 2007). However, another study conducted in mouse ES cells concluded that, although the first steps may be shared, alternative NHEJ in ES cells may be distinct from SSA during the late steps of repair (Bennardo et al., 2008). This conclusion came from the observation that mouse RAD52 was not able to stimulate alternative NHEJ in their experimental chromosomal context although the protein was able to promote SSA when the entire coding sequence of GFP was involved in homology-directed repair (Bennardo et al., 2008). One possibility however, would be that the annealing process may require another protein than RAD52, similarly, to the situation in budding yeast where RAD59, a RAD52 homolog, is required for annealing when only very short homologous sequences are available for SSA (Sugawara et al., 2000). In support of this, observations in *RAD52* knock-out mouse models suggested that mouse RAD52 may only be involved in certain types of DSB repair processes while other HR-dependent events may be catalyzed by distinct proteins functionally related to RAD52 (Rijkers et al., 1998). This remains to be tested experimentally.

Additional proteins, whether from yeast or from higher eukaryotes, were reported to play a role in MMEJ. POL4, a member of the PolX family of polymerases with gap-filling activity, and proteins from the mismatch repair pathways were found to be required for MMEJ-dependent repair of substrates with non-perfect microhomologies in fission yeast (Decottignies, 2007). The MRE11 complex was found to be required for MMEJ in budding yeast (Ma et al., 2003), *Arabidopsis* (Heacock et al., 2004) and human cells (Delia-Maria et al., 2011), but dispensable for fission yeast MMEJ using an extrachromosomal DSB repair assay (Decottignies, 2007). It is believed however, that fission yeast MRE11 complex may be required for MMEJ events in a chromosomal context and/or for intermolecular MMEJ-dependent ligations (Decottignies, 2005, 2007). As stated above, first evidences for the involvement of LIG3 in the MMEJ process were provided by biochemical fractionation of *X. laevis* egg extracts (Gottlich et al., 1998). LIG3 contribution to MMEJ was later confirmed in HeLa cells (Wang et al., 2005), in human HTD114 cell line (Liang et al., 2008) and in mice (Simsek et al., 2011).

In mature mouse B cells activated by antigens, recent *in vivo* evidences indeed support the existence of a powerful backup mechanism able to compensate for NHEJ during immunoglobulin class switch recombination (CSR; Soulard-Sprauel et al., 2007; Yan et al., 2007). Whether this *in vivo* backup mechanism is similar to the MMEJ repair pathway described above is however still a matter of debate. In favor of this hypothesis, the backup CSR activity detected in the absence of either XRCC4 or LIG4 was found to operate through the recognition of microhomologies at DNA break borders and, in agreement with two previous reports (Audebert et al., 2004; Wang et al., 2005), was proposed to rely on XRCC1/LIG3 complex (Yan et al., 2007). Interestingly, XRCC1 was previously involved in SSA (Stark et al., 2004), further

supporting the view that alternative NHEJ may similarly operate through a micro-SSA-like mechanism in immune cells. A more recent study published by the group of Jasen reported that, similarly to what happens in human cells in culture, mouse LIG3 is involved in an alternative end-joining pathway operating through annealing at pre-existing microhomologies (Simsek et al., 2011). They proposed that LIG4 was acting as a repressor of the DNA end resection activity required to produce the complementary ssDNA ends.

Although the studies reported above in various eukaryotic species converged onto the identification of the alternative end-joining backup pathway as a microhomology-dependent mechanism presumably relying on LIG3, recent data led to revise this view. Indeed, experiments performed in mammalian cells suggested the existence of an additional alternative end-joining pathway presumably relying on Ligase I (LIG1) and able to repair DSBs independently of pre-existing microhomologies (Boboila et al., 2010a,b, 2012; Simsek et al., 2011). First *in vivo* evidences came from the observation that, in either *KU70*^{-/-} or *KU70*^{-/-}/*LIG4*^{-/-} mice, CSR appears to operate through two distinct alternative end-joining mechanisms in B cells, with only one relying on microhomologies (Boboila et al., 2010a). This newly unraveled alternative end-joining mechanism was not initially detected in either *LIG4*^{-/-} or *XRCC4*^{-/-} mouse B cells for which microhomologies were recovered at all CSR junctions, suggesting that it may be repressed by Ku70/Ku80. Further support in favor of the existence of a second alternative end-joining mechanism not relying on pre-existing microhomologies was provided by sequencing of chromosomal translocation breakpoints recovered in B cells from *KU70*^{-/-} mice (Boboila et al., 2010b). Shortly after, using an experimental system of chromosomal translocation induction based on zinc finger nuclease-induced DSBs in mouse cells, the group of Jasen reported that, while translocation breakpoints displayed less microhomologies in the absence of LIG3, LIG1 depletion did not affect microhomology use (Simsek et al., 2011). Note however that an *in vitro* study performed in human HTD114 cell line reported that both LIG1 and LIG3 were involved in MMEJ-dependent repair of an extrachromosomal DSB, although contribution of LIG3 appeared to be more important (Liang et al., 2008).

Altogether, data suggest the possible existence of two distinct alternative end-joining repair processes, both repressed by Ku70/Ku80 (**Figure 2**). The first one appears to rely on the presence of microhomologies for repair and I propose that it operates through a micro-SSA-type mechanism and involves LIG3. The second pathway of alternative end-joining does not appear to depend on pre-existing microhomologies and is believed to rely on LIG1. However, evidences for the conservation of the latter pathway throughout the eukaryotic lineage are still lacking.

IMPLICATION OF ALTERNATIVE NHEJ IN TELOMERE FUSION EVENTS

Telomeres at the end of linear chromosomes are reportedly resistant to end-to-end fusions thanks to the binding of so-called shelterin proteins (van Steensel et al., 1998). Accordingly, when normal shelterin density is breached, protection

of telomeres against DNA damage activation is no longer ensured and DNA repair enzymes have access to telomeric DNA. Hence, loss of TRF2 shelterin component at mammalian telomeres induces telomere deprotection and LIG4-dependent fusions (Smogorzewska and de Lange, 2002). Further experiments revealed a direct role for mammalian RAP1/TRF2 complex in protection of telomeric DNA from NHEJ, independently of the involvement of TRF2 in telomeric-loop formation (Bae and Baumann, 2007).

Although NHEJ is clearly able to mediate end-to-end fusions in a telomere-deficient background, this DNA repair pathway is not required to catalyze all types of telomere fusions. Indeed, telomerase-deficient fission yeast mutants lacking either Ku70 or LIG4 display rearranged telomeres and chromosome circularization, indicating that alternative end-joining mechanisms are able to promote telomere fusion (Baumann and Cech, 2000). Similar conclusions were subsequently drawn from studies in budding yeast (Mieczkowski et al., 2003) and *Arabidopsis* (Riha and Shippen, 2003). A molecular analysis was then published in which authors analyzed telomere fusion events in *Arabidopsis* mutants lacking both TERT catalytic subunit of telomerase and Ku70 DNA repair protein (Heacock et al., 2004). Fusions between telomeric and subtelomeric regions of plant chromosomes were associated with large deletions, extending to more than 300 bp, and displayed overlapping homologies of up to 12 bp. Here too, Ku70 was acting as a strong inhibitor of the MMEJ-dependent mechanism of telomere fusion while MREll was found to promote fusions (Heacock et al., 2004). Subsequent work by the same group revealed that, as expected from an alternative end-joining mechanism, LIG4 was not required for plant telomere fusions (Heacock et al., 2007). Studies in human cells revealed similar mechanisms of telomere fusion in cells forced to divide in the absence of telomerase. In these cells, telomere fusions occurred with concomitant deletion of one or both telomeres and were characterized by the presence of microhomologies (Capper et al., 2007; Letsolo et al., 2010).

Following these *in vitro* studies, a report provided evidences for the involvement of both NHEJ and MMEJ repair pathways in mouse telomere fusion events *in vivo* (Rai et al., 2010). Using a combination of mutants and shRNA constructs, the authors showed that, while TRF2/RAP1 complex protects telomeres from ATM activation and NHEJ, single-stranded telomeric DNA-binding protein POT1, in conjunction with TPP1 shelterin component, inhibits ATR activation and alternative NHEJ. In agreement with previous data in human cells, their work further suggested that alternative NHEJ is the main pathway to process dysfunctional telomeres in mouse cells experiencing natural telomere erosion (Rai et al., 2010). Hence, despite a strong protection against NHEJ provided by TRF2, mammalian telomeres can be targets of MMEJ. Elegant demonstration of the role of shelterin components in telomere end protection was recently provided by the group of de Lange (Sfeir and de Lange, 2012). They confirmed the role of TRF2 as repressor of both ATM signaling and classical NHEJ and the role of POT1 in ATR signaling repression. They also showed that alternative NHEJ was repressed by various shelterin components as well as by Ku70/Ku80 and proposed that the redundancy of repressors may ensure better protection

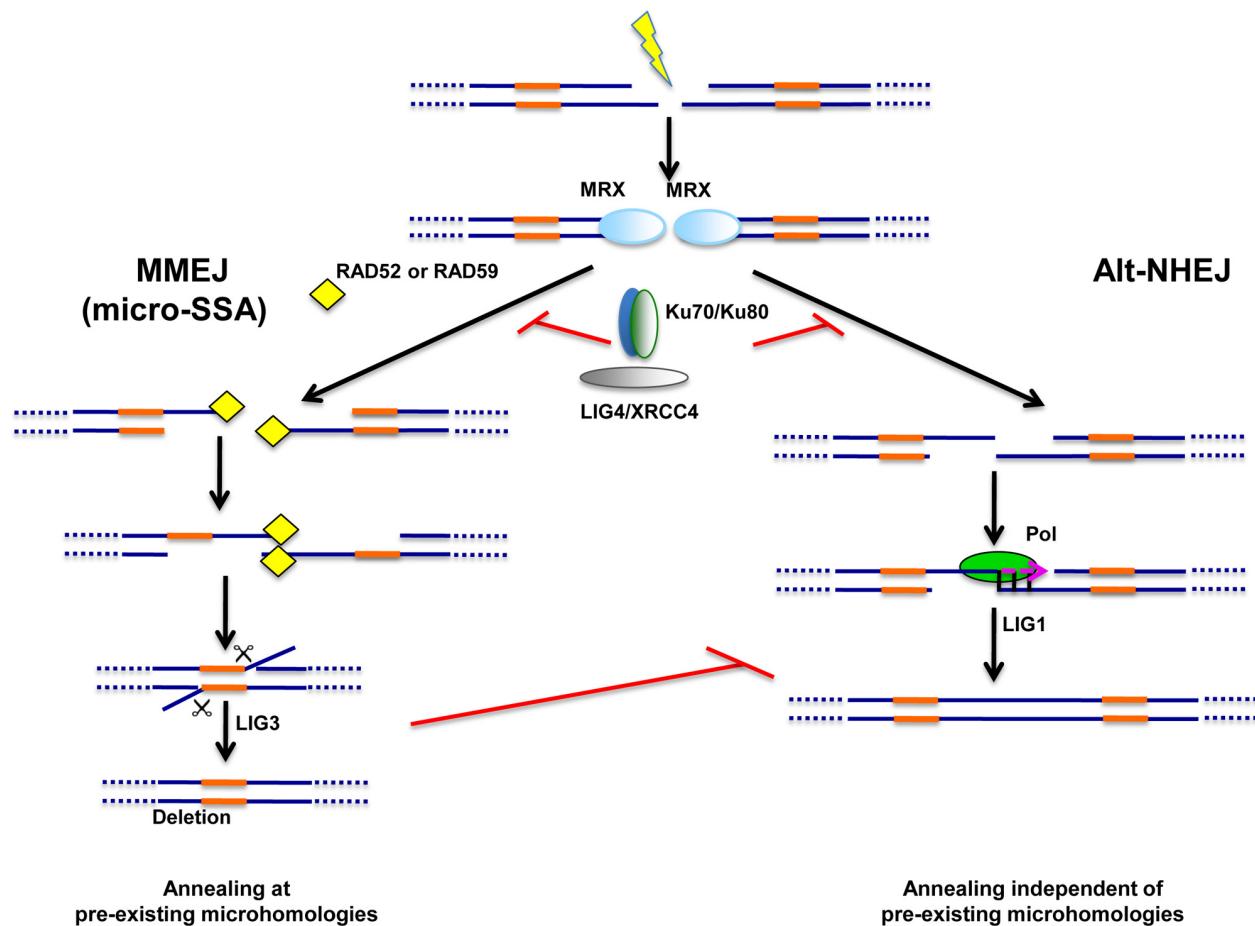


FIGURE 2 | Alternative end-joining pathways of DNA repair. Two types of alternative end-joining pathways of DSB repair were unraveled in NHEJ-deficient cells. One pathway, dubbed “microhomology-mediated end-joining” or MMEJ, relies on pre-existing microhomologies around the break (in orange) and is likely to operate through a mechanism related to single strand-annealing (micro-SSA). MMEJ appears to rely on Ligase III (LIG3) for sealing. Unlike MMEJ, the second alternative pathway, dubbed Alt-NHEJ, does not require the presence of pre-existing microhomologies

and may rather rely on Ligase I (LIG1). Simsek et al. (2011) proposed that microhomologies may nevertheless be generated by a polymerase activity (Pol) operating at one DNA end. The same study suggested that LIG1 may only function in the absence of LIG3 as a back-up ligase, at least in mouse cells. Both MMEJ and Alt-NHEJ are repressed by the NHEJ machinery (Ku70/Ku80, LIG4/XRCC4). The MRX complex is likely to play important roles for both alternative pathways during the first steps of repair.

against dangerous alternative NHEJ at telomeres (Sfeir and de Lange, 2012).

Hence, the above studies clearly pointed toward an important contribution of alternative NHEJ to pathologic chromosome fusion events in cells with dysfunctional telomeres. Although evidences for an involvement of SSA proteins in these end-joining events is still lacking, telomere fusions are characterized by microhomologies at junctions, are repressed by Ku70/Ku80 and rely on the MRE11 complex in plants and possibly also in human cells (Tankimanova et al., 2012).

MUTAGENIC POTENTIAL OF ALTERNATIVE NHEJ IN MAMMALS

In the late 1990s, it became evident that NHEJ acts as a tumor suppressing mechanism. Indeed, mice lacking both p53 and a NHEJ component, like DNA-PKcs, Ku80, XRCC4, or LIG4, were found to die in early postnatal life due to an elevated frequency of B cell

lymphomas displaying *IgH-Myc* translocations and amplifications (reviewed in Sharpless et al., 2001). Importantly, these lymphomas were qualitatively distinct from those arising in a p53-deficient background alone as, in the latter mouse mutants, tumors had a later onset and did not generally harbor translocations. Following these observations, *IgH-Myc* translocation junctions were recovered from *XRCC4*^{-/-}/*p53*^{-/-} mice in order to characterize the DNA repair mechanisms involved in chromosomal translocations. Sequencing of breakpoint junctions revealed the presence of microhomologous DNA sequences (Zhu et al., 2002; Wang et al., 2008). *LIG4* haploinsufficiency was also reported to increase sarcoma formation in *INK4a/ARF*^{-/-} mice by inducing chromosomal translocations, amplifications and deletions but translocation junctions were not characterized (Sharpless et al., 2001). In a more recent report, the group of F. Alt confirmed that an alternative end-joining pathway robustly catalyzes translocations in *KU70*^{-/-}/*LIG4*^{-/-} mice B cells that are fully deficient

for classical NHEJ (Boboila et al., 2010b). However, as the authors did not detect any bias toward MMEJ at breakpoint junctions, they suggested that translocations were mediated by an alternative end-joining mechanism not relying on microhomologies. It should be tested whether this mutagenic alternative end-joining mechanism operating in B cells of *KU70^{-/-}* mice requires LIG1.

In human, an analysis of high-grade bladder carcinomas suggested that MMEJ may contribute to the high genomic instability of bladder cancer (Bentley et al., 2004). Indeed, authors showed that these tumors were highly proficient in their ability to perform MMEJ, even though Ku, DNA-PKcs and XRCC4 proteins were expressed at normal level.

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Altogether, data reported so far indicate that, although the classical LIG4/Ku-dependent NHEJ pathway appears to act as a potent tumor suppressor mechanism, alternative end-joining pathways, whether relying on microhomologies or not, promote chromosomal translocations. In the future, it would be interesting to better characterize these alternative pathways of end-joining and to identify the genes involved in the repair processes.

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MUTYH DNA glycosylase: the rationale for removing undamaged bases from the DNA

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Maintenance of genetic stability is crucial for all organisms in order to avoid the onset of deleterious diseases such as cancer. One of the many proveniences of DNA base damage in mammalian cells is oxidative stress, arising from a variety of endogenous and exogenous sources, generating highly mutagenic oxidative DNA lesions. One of the best characterized oxidative DNA lesion is 7,8-dihydro-8-oxoguanine (8-oxo-G), which can give rise to base substitution mutations (also known as point mutations). This mutagenicity is due to the miscoding potential of 8-oxo-G that instructs most DNA polymerases (pol) to preferentially insert an Adenine (A) opposite 8-oxo-G instead of the appropriate Cytosine (C). If left unrepaired, such A:8-oxo-G mispairs can give rise to CG→AT transversion mutations. A:8-oxo-G mispairs are proficiently recognized by the MutY glycosylase homologue (MUTYH). MUTYH can remove the mispaired A from an A:8-oxo-G, giving way to the canonical base-excision repair (BER) that ultimately restores undamaged Guanine (G). The importance of this MUTYH-initiated pathway is illustrated by the fact that biallelic mutations in the *MUTYH* gene are associated with a hereditary colorectal cancer syndrome termed MUTYH-associated polyposis (MAP). In this review, we will focus on MUTYH, from its discovery to the most recent data regarding its cellular roles and interaction partners. We discuss the involvement of the MUTYH protein in the A:8-oxo-G BER pathway acting together with pol λ, the pol that can faithfully incorporate C opposite 8-oxo-G and thus bypass this lesion in a correct manner. We also outline the current knowledge about the regulation of MUTYH itself and the A:8-oxo-G repair pathway by posttranslational modifications (PTM). Finally, to achieve a clearer overview of the literature, we will briefly touch on the rather confusing MUTYH nomenclature. In short, MUTYH is a unique DNA glycosylase that catalyzes the excision of an undamaged base from DNA.

Keywords: MUTYH, MUTYH-associated polyposis (MAP), MYH, mutY, DNA polymerase beta and lambda, base-excision repair (BER), DNA glycosylases, 8-oxo-guanine

INTRODUCTION

Cellular DNA is constantly under attack of damaging agents, such as reactive oxygen species (ROS), that derive from a multitude of exogenous and endogenous sources (reviewed in Van Loon et al., 2010). One of the main consequences of ROS impact on DNA is the formation of 8-oxo-G, a frequent DNA lesion estimated to arise around 1000–7000 times per cell per day (Collins, 1999; European Standards Committee on Oxidative DNA Damage (ESCODD), 2003; Gedik and Collins, 2005; Friedberg, 2006). To counteract this heavy burden of 8-oxo-G lesions, a multi-component system involving a plethora of enzymes has evolved both in bacteria and mammals. 8-oxo-dGTP, which arises upon oxidation of the nucleotide pool, is hydrolyzed by the enzymes MutT/MTH1, which therefore prevent incorporation of 8-oxo-dGTP into nascent DNA. When a C:G base pair is oxidized to C:8-oxo-G, the enzyme Fpg (also known as MutM)/OGG can catalyze the removal of 8-oxo-G from these base pairs. Furthermore, other proteins such as the mismatch-repair pathway component MutS/MSH2, or the Nei endonuclease VIII/NEIL1 and NEIL2 have been shown to protect the genome from the mutagenic

consequences of 8-oxo-G damage. Finally, A:8-oxo-G base pairs are a substrate for MutY/MUTYH, which is the protein in the focus of this review. Information on the contribution of all of the other factors to genetic stability can be found in these detailed reviews (Lu et al., 2006a; Tsuzuki et al., 2007).

In the *syn* conformation, 8-oxo-G functionally mimics the base pairing properties of a Thymine (T), which leads to the formation of stable A(*anti*):8-oxo-G(*syn*) Hoogsteen base pairs (David et al., 2007). Due to this particular behavior of 8-oxo-G, most pols often bypass 8-oxo-G lesions inaccurately by incorrectly inserting an A instead of the correct C, therefore giving rise to A:8-oxo-G mismatches (Maga et al., 2007). If these A:8-oxo-G mismatches are not repaired before the next round of replication, they can generate CG→AT transversion mutations that have the potential to transform cells and lead to cancer (Greenman et al., 2007). Oxidative damage to C:G base pairs in DNA leads to the generation of C:8-oxo-G base pairs. The majority of 8-oxo-G from these base pairs is recognized and removed from the genome by the OGG1 DNA glycosylase, which initiates a canonical short-patch base-excision repair (SP-BER) pathway

involving apurinic endonuclease 1 (APE1), pol β , XRCC1, and DNA ligase III. This results in the restoration of the original C:G base pair [see **Figure 1**, Dianov et al., 1998; Fortini et al., 1999; Pascucci et al., 2002; Fromme et al., 2003 and reviewed in Van Loon et al. (2010)]. However, a problematic situation may arise when the replication fork encounters an 8-oxo-G. Such a scenario can result from either a failure of OGG1 to repair all 8-oxo-G lesions before the start of replication, or from oxidative stress during the S-phase. In contrast to UV-induced lesions, for instance, that present a block to the replicative pols (reviewed in Lehmann, 2002), 8-oxo-G is not considered a blocking lesion *per se* (Shibutani et al., 1991; Mozzherin et al., 1997; Avkin and

Livneh, 2002). Nevertheless, it has been found that replicative pols (such as the Klenow fragment of *E. coli* pol I, calf thymus pol α and pol δ) show transient inhibition of chain extension 3' to 8-oxo-G and extend promutagenic A:8-oxo-G base pairs more efficiently than the correct C:8-oxo-G base pairs (Shibutani et al., 1991; Einolf and Guengerich, 2001). Also, human pol δ has been demonstrated to stall at sites of 8-oxo-G lesions (Fazlieva et al., 2009). Very recently, we have proposed that a switch between the replicative pol δ and the repair pol λ promotes the correct bypass of 8-oxo-G lesions during replication (Markkanen et al., 2012a). Nevertheless, oxidative stress in context of DNA replication can result in the generation of A:8-oxo-G mispairs, which are

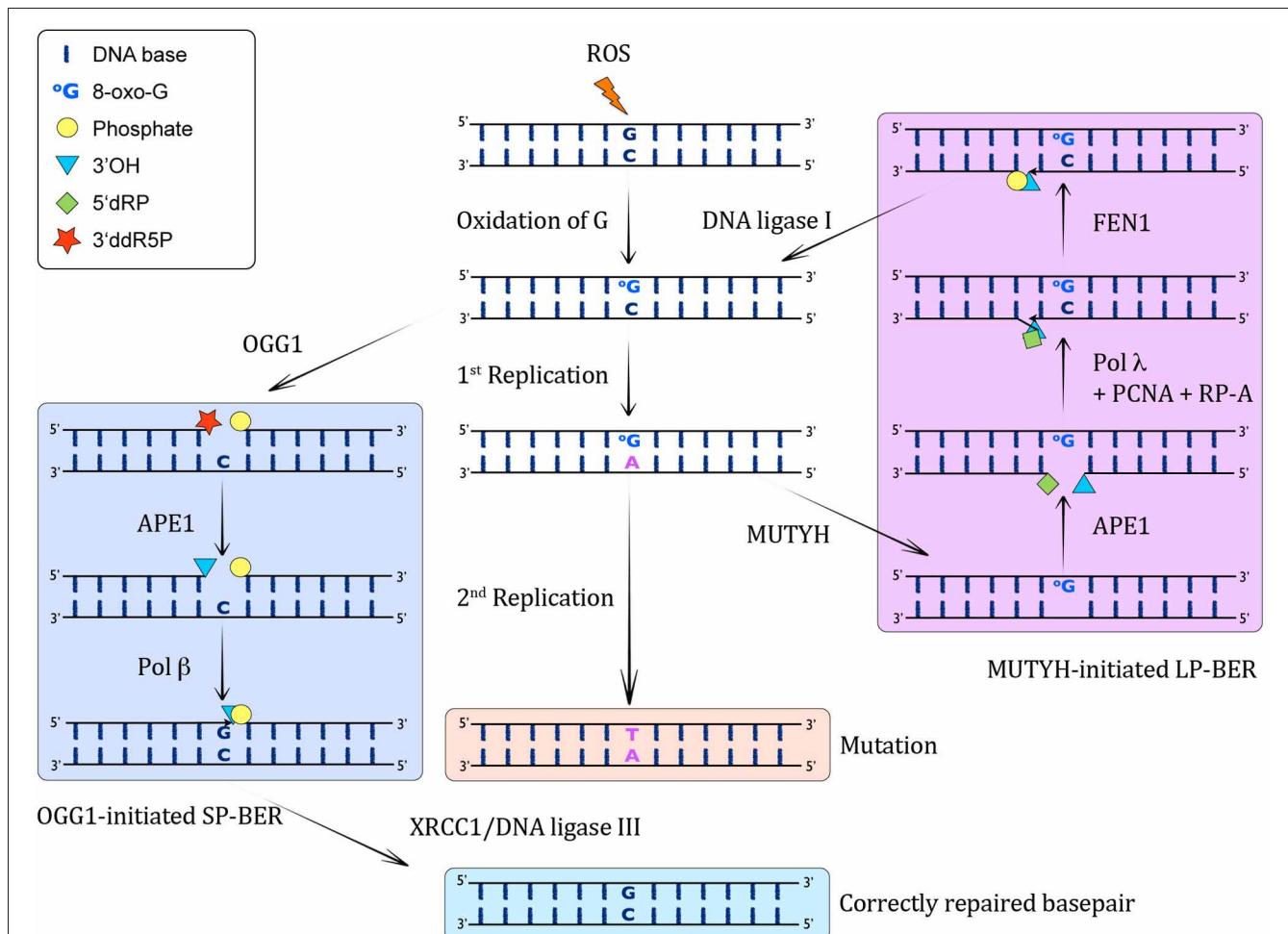


FIGURE 1 | MUTYH-initiated BER of A:8-oxo-G lesions. When ROS attack DNA, they lead to the formation of C:8-oxo-G base pairs through oxidation of G. **Left column:** These can be recognized by OGG1, which excises the 8-oxo-G and incises the resulting AP-site by β -elimination, giving rise to a 3'ddR5P and a 5'P residue. This 3' sugar phosphate is then removed by APE1, yielding in a 1 nucleotide gap with a 3'OH and a 5'P. Subsequently, pol β catalyzes the insertion of a G opposite the templating C in this SP-BER pathway, and ligation by XRCC1/DNA ligase I leads to restoration of an intact, correctly base-paired double-stranded DNA again. **Middle column:** If the C:8-oxo-G base pairs are not recognized before S-phase by OGG1, or they arise through oxidation in S-phase, the replicative pols will often incorporate a wrong A opposite 8-oxo-G, giving rise to A:8-oxo-G mispairs. If these are not

corrected, another round of replication will lead to a CG \rightarrow AT transversion mutation. **Right column:** The A:8-oxo-G base pairs can be recognized by MUTYH, which catalyzes the excision of the wrong A from opposite 8-oxo-G, leading to the formation of an AP site. This AP site is further processed by APE1, which results in a 1 nt gap with 3'OH and 5'P moieties. The incorporation of the correct C opposite 8-oxo-G and one more nucleotide is performed by pol λ in collaboration with the cofactors PCNA and RP-A, thus performing strand displacement of the downstream DNA strand. FEN1 cleaves the 5' flap, leading to a 5'P moiety, which can be ligated by DNA ligase I to yield an intact C:8-oxo-G containing double-stranded DNA. This C:8-oxo-G is then again substrate for OGG1-mediated removal of 8-oxo-G (**left column**).

substrates for MUTYH. As a monofunctional DNA glycosylase, MUTYH catalyzes the excision of the A mispaired with 8-oxo-G. Thus, MUTYH is a unique glycosylase as far as it removes an *undamaged* base from *opposite a DNA lesion*, instead of removing the damaged base. The steps following MUTYH-initiated repair of A:8-oxo-G lesions are discussed in more detail in the following. As this review is focused on MUTYH, the interested reader is referred to a detailed excellent review for more information on the cellular DNA glycosylases in general (Jacobs and Schar, 2012).

DISCOVERY

MutY, along with the other 8-oxo-G repair enzymes FpG and MutT, is phylogenetically an ancient protein, emphasizing the importance to cope correctly and efficiently with oxidative damage for living organisms (Jansson et al., 2010). MutY homologues have been identified in many organisms, both in prokaryotes as well as in eukaryotes. They all share the unique function of being able to remove an A that is incorrectly paired with 8-oxo-G, G, C, 5-hydroxyuracil (5-OH-U), or 2-hydroxyadenine (2-OH-A), as specified later on.

DISCOVERY OF MutY IN *E. coli*

The first mutators in *E. coli* strains were described about 60 years ago (Trefters et al., 1954) based on the observation that some strains showed an altered antibiotic resistance. These findings were used to engineer a systematic screening for mutators with certain properties. Nghiem et al. used Lac⁻ *E. coli* strains transformed with constructs encoding for β-galactosidase, each inactivated by a specific point mutation. When reverted back to Lac⁺ the specific base substitution reactivating the β-galactosidase could be identified. A strain with an increase in C:G→A:T transversion mutations revealed the so far not described locus called *mutY* to be responsible for the observed mutator phenotype (Nghiem et al., 1988).

In addition to the *mutY*, another locus, called *mutM*, was found to cause a change from C:G→A:T (Cabrera et al., 1988) when mutated and was later identified to encode the formamidopyrimidine DNA glycosylase (Fpg) (Michaels et al., 1991). Neither *mutY* nor *mutM* strains showed a very pronounced phenotype on their own, but double mutant strains expressed an extremely high mutation rate (Michaels et al., 1992a). Mutations in *mutY* and *mutM* exclusively enhanced one type of transversion mutation, while neither frameshifts nor deletions were found, in contrast to what had been reported for other mutators (Nghiem et al., 1988).

It had been shown that the correction of A:G mispairs in *E. coli* extracts could occur by two distinct pathways: the methylation-dependent mutHLS mismatch-repair pathway that recognizes a variety of mismatches and repairs the unmethylated DNA strand, and a second methylation-independent mechanism specific to A:G mismatches (Su et al., 1988). Analysis of the second pathway revealed that the *mutY* gene product was involved in this novel DNA repair mechanism (Au et al., 1988). Cells defective in the mutHLS-dependent repair but proficient for *mutY* were still able to prevent C:G→A:T transversion mutations, and the *mutY*-dependent repair was dominant if both pathways were available. The function of the *mutY* gene product was finally elucidated by

purification of a protein according to its ability to repair a A:G mismatch. The 36 kDa protein was capable of removing the mispaired base A from dsDNA and rendered the strand sensitive for cleavage by apurinic/apyrimidinic endonucleases at the site of the mismatch (Au et al., 1989). This result further underlined the hypothesis that *mutY* encoded for a DNA glycosylase, termed MutY, that initiated the repair of A:G mismatches while other mispairs, as for example A:C, were not recognized. Further on, Su et al. showed that MutY, with help of pol I and DNA ligase, was able to restore specifically A:G mismatches to C:G in a sequence independent manner (Su et al., 1988). Cloning and sequencing of the *mutY* gene finally revealed that it encoded for a 350 amino acids DNA glycosylase that could rescue the mutator phenotype of *mutY E. coli* strains (Michaels et al., 1990).

DISCOVERY OF THE MAMMALIAN MutY HOMOLOG (MUTYH)

The first experiments using cell extracts showed that, in general, humans had a repair mechanism for mismatches similar to those of bacteria preventing the generation of mutations during replication (Holmes et al., 1990; Thomas et al., 1991). The analysis of human HeLa nuclear extracts revealed the existence of two enzyme systems that could nick DNA specifically at sites of mispaired bases (Yeh et al., 1991). One of the identified systems showed a specific substrate recognition, cleaving the DNA at A:G mismatches and could be separated from other enzymes by chromatography. Since this enzyme showed the same substrate specificity as the bacterial MutY, Yeh et al. proposed to have identified its human homologue (Yeh et al., 1991).

The first characterization of a mammalian homologue of MutY was published by McGoldrick et al., who purified an enzyme from calf thymus that was acting on A:G mismatches. Apart from the substrate specificity they described several other features indicating that they had indeed purified a MutY homologue: An AP endonuclease activity was co-purified with the DNA glycosylase and the antibody generated against bacterial MutY recognized a band at the expected size and could inhibit the DNA glycosylase activity of the purified protein (McGoldrick et al., 1995). Based on the finding that CG→AT transversion mutations occur often in different kinds of cancer (Hollstein et al., 1991), the authors already hypothesized that the human MutY homologue might be involved in cancer prevention.

A few years after the characterization of human homologue of the 8-oxo-dGTP hydrolase MutT which removes 8-oxo-dGTP from the nucleotide pool (Sakumi et al., 1993), Slupska et al. succeeded in cloning and sequencing of the human *mutY* gene, termed *MUTYH* (Slupska et al., 1996). By screening different cDNA libraries for amino-acid sequence homologies, they identified a gene that showed 41% identity with the *E. coli* *mutY*. The gene was 7.1 kb long, contained 15 introns and encoded for a protein of 535 amino acids in length, which was consistent with the size of the protein that had been detected in HeLa cells (McGoldrick et al., 1995). By using *in situ* hybridization they could map the gene on chromosome 1, between *p32.1* and *p34.3*. The current status of knowledge is that the human *MUTYH* gene codes for at least 10 different isoforms of *MUTYH* protein. There are three major transcripts, α , β , and γ that differ from each other in the 5' end sequence and are generated through alternative

splicing (Ohtsubo et al., 2000). The transcript $\alpha 3$ was found to be the originally identified MUTYH, but so far it is not entirely clear what the functions of the different isoforms are and to which cell compartment they are localized, as we will discuss below in more detail.

NOMENCLATURE OF MUTYH

Currently, literature referring to the protein product of the mammalian *MUTYH* gene is rather confusing due to a diversity of different synonyms and writing styles that have been used over the last years. The most commonly used names are MUTYH, MutYH, MYH, and hMYH. Here, we propose to uniformly use MUTYH as name for this protein in mammals in order to simplify the literature overview, because of the following reasons. Firstly, *MUTYH* [*MutY homolog (E. coli)*] is the officially approved name for the gene from which MUTYH derives (HUGO Gene Nomenclature Committee). Secondly, the official protein name listed by leading protein databases (UniProtKB, neXtProt, Ensembl, and Reactome) is MUTYH. Thirdly, as the protein derives its name from the bacterial homolog *mutY* that was discovered first, the logical extension would be the addition of an “H” for “homolog” at the end of the protein name, which also leads to easy recognition of homology between MUTYH and MutY.

FUNCTION OF MutY AND MUTYH

MutY

MutY—substrate specificity

The currently known substrates for MutY and MUTYH are summarized in **Table 1**. Analysis of the substrate specificity for MutY demonstrated that it acts as a glycosylase on A:G, A:8-oxo-G, A:C, and A:8-oxo-A mismatches, always removing the undamaged A from each substrate (Michaels et al., 1992b). Lu et al. further refined the DNA determinants and substrate specificities for the catalytic activity of MutY, using binding and endonuclease assays with a variety of different A-containing mismatches, and concluded that DNA sequences proximal to the mismatch as well as specific functional groups of mismatched bases dictate the recognition and catalysis by MutY (Lu et al., 1995). Moreover, while MutY bound the A:8-oxo-G much tighter than A:G, its activity on A:8-oxo-G was weaker than on A:G mismatches. Bulychev et al. contradicted this notion in a subsequent report stating that A:8-oxo-G appeared to be the natural substrate for MutY, as judged by the specificity constants and the fact that the presence of an 8-oxo-group in G increased significantly the rate of removal of A from all tested substrates (Bulychev et al., 1996). Additionally to A:8-oxo-G, MutY was shown to bind to G:8-oxo-G mismatches as well, and it was capable of removing G from this substrate (Zhang et al., 1998). The sequence context surrounding an A:G mismatch was shown to also significantly influence the catalytic activity of MutY (Sanchez et al., 2003).

8-oxo-G is chemically labile toward further oxidation into guanidinoimidodihydantoin (Sp1), spiroiminodihydantoin (Sp2), oxaluric acid, and urea. Delaney et al. investigated the activity of MutY on these lesions by introducing them into single-stranded viral genomes which were replicated in *E. coli* proficient or deficient for MutY (Delaney et al., 2007). These lesions were found to be equally mutagenic in terms of frequency in both genetic

Table 1 | Substrate specificities of the different MutY and MUTYH proteins.

Protein	Base pair substrate	Excised base	References
<i>MutY E. coli</i>	A:G	A	Michaels et al., 1992b; Lu et al., 1995; Gogos et al., 1996; Noll et al., 1999; Gu and Lu, 2001
	A:8-oxo-G	A	Michaels et al., 1992b; Lu et al., 1995; Gogos et al., 1996; Noll et al., 1999; Gu and Lu, 2001
	A:C	A	Michaels et al., 1992b
	A:8-oxo-A	A	Michaels et al., 1992b
	2-OH-A:G	2-OH-A	Hashiguchi et al., 2002; Pope and David, 2005
	2-OH-A:8-oxo-G	2-OH-A	Pope and David, 2005
	A:FapyG	A	Wiederholt et al., 2003
<i>MutY Th. thermophilus</i>	G:8-oxo-G	G	Zhang et al., 1998
	A:8-oxo-G	A	Back et al., 2006
	A:G	A	Back et al., 2006
	G:8-oxo-G	G	Back et al., 2006
<i>MUTYH S. pombe</i>	T:8-oxo-G	T	Back et al., 2006
	G:8-oxo-G	G	Doi et al., 2005
	A:8-oxo-G	A	Doi et al., 2005
<i>MUTYH mouse</i>	A:8-oxo-G	A	Tominaga et al., 2004; Pope and David, 2005
	A:G	A	Pope and David, 2005
	2-OH-A:G	2-OH-A	Pope and David, 2005
	2-OH-A:8-oxo-G	2-OH-A	Pope and David, 2005
<i>MUTYH calf</i>	A:G	A	McGoldrick et al., 1995; Parker et al., 2000
	A:8-oxo-G	A	McGoldrick et al., 1995; Parker et al., 2000
	A:C	A	McGoldrick et al., 1995; Parker et al., 2000
	G:8-oxo-G	G	Parker et al., 2000
	T:8-oxo-G	T	Parker et al., 2000
	C:8-oxo-G	C	Parker et al., 2000
<i>MUTYH human</i>	A:8-oxo-G	A	Slupska et al., 1999; Shinmura et al., 2000; Gu and Lu, 2001
	A:G	A	Slupska et al., 1999; Shinmura et al., 2000; Gu and Lu, 2001
	2-OH-A:G	2-OH-A	Ushijima et al., 2005

backgrounds and to yield similar mutation spectra, suggesting that MutY does not play a role in the excision of these bases. Interestingly Sp1 and Sp2 were more toxic to the cells that were proficient in MutY.

2-hydroxyadenine (2-OH-A) is a lesion that is induced by Fenton-type ROS and is produced for instance by H₂O₂ treatment of cultured mammalian cells (Jaruga and Dizdaroglu, 1996). Incorporation of 2-OH-dATP into the bacterial genome by pol III was shown to yield slightly increased mutant frequencies in a MutY deficient background in *E. coli*, suggesting that the processing of 2-OH-A damage possibly also involves the action of MutY (Kamiya and Kasai, 2000a). However, follow-up work by the same authors showed that, irrespectively of the base in the complementary strand, DNA with 2-OH-A presented a very poor substrate for MutY, and therefore illustrated that neither MutY nor Fpg seemed to play a role in 2-OH-A removal from DNA (Kamiya and Kasai, 2000b). Another result by Hashiguchi et al. again reassessed this finding and they reported that MutY indeed bound to 2-OH-A in duplex with G, A, or C and displayed a DNA glycosylase activity capable of removing 2-OH-A from 2-OH-A:G mismatches, which was dependent on the C-terminal domain of the protein (Hashiguchi et al., 2002).

FapyG is a DNA lesion that arises from oxidative stress by ring fragmentation of the purine base. MutY excised A from A:FapyG mismatches, and this reaction was faster than the removal of A from A:G, but still slower than that from A:8-oxo-G *in vitro* (Wiederolt et al., 2003).

One group reported that MutY efficiently recognized 7-deaza-2'-deoxyadenosine (Z) and its non-polar isostere 4-methylindole-beta-deoxynucleoside (M) opposite 8-oxo-G and G in DNA, with a preference for M:8-oxo-G over Z:8-oxo-G mispairs (Chepanoske et al., 2000b). This finding was contradicting a previous report, in which Z:G mispairs were neither bound nor processed by MutY (Lu et al., 1995).

Lu et al. showed that MutY competes with and inhibits endonuclease VIII on its natural substrate, the hydroxyurea (hU):A mismatch (Lu et al., 2006b).

A MutY variant from *Thermus thermophilus* processed A:8-oxo-G, G:8-oxo-G as well as T:8-oxo-G and A:G mismatches, but in contrast to other MutY variants, was shown to harbor a bifunctional glycosylase activity (Back et al., 2006).

MutY—enzymatic activity

The cloning of *E. coli* MutY revealed that it shared significant sequence homology to the bacterial endonuclease III (EndoIII), which acts on damaged base pairs (Michaels et al., 1990). MutY was shown to be an iron-sulfur (Fe-S) cluster protein containing both N-glycosylase and a 3' AP endonuclease activity (Tsai-Wu et al., 1992). Initially there was some confusion regarding the enzymatic activity of MutY. While some reports stated that MutY also acted as an endonuclease on AP sites, therefore functioning as a bifunctional glycosylase (Tsai-Wu et al., 1992; Lu et al., 1995, 1996; Gogos et al., 1996; Manuel and Lloyd, 1997), Zharkov and Grollman showed that MutY does not harbor any AP lyase activity (Zharkov and Grollman, 1998). They hypothesized that the previous observations for the observed AP-activity were rather caused by heat-induced cleavage of the AP site and not due to an actual enzymatic activity. Moreover, this report suggested that the tight binding of MutY to its DNA substrate prevented the access of another bacterial glycosylase, the formamidopyrimidine-DNA glycosylase (Fpg), to the substrate. Consequently, MutY seemed

to prevent a possible generation of a DNA double-strand break (DSB) by Fpg and thus possibly to play a role in the regulation of BER.

MutY—catalytic mechanism

When considering the catalytic activity of MutY (or any other DNA glycosylase), it is important to keep in mind that the catalytic cycle can be roughly subdivided into different stages, namely (1) recognition and binding of the enzyme to the substrate, (2) hydrolysis of the N-glycosidic bond or base-excission, and (3) dissociation of the enzyme or release of the resulting AP site. We have tried to structure the discussion according to these three steps in the catalytic cycle, whenever possible.

Substrate recognition. Multiple studies elucidating the contributions of the different parts of the MutY protein have been undertaken. Proteolytic digestion of MutY with thermolysin produced two fragments, an N-terminal one of 25 kDa and a C-terminal one of 12 kDa, respectively (Gogos et al., 1996). While the 12 kDa fragment did not display any detectable enzymatic activity, it was found to play an important role in the repair of mismatched oxidized DNA, as its deletion significantly impaired the binding and activity of MutY on A:8-oxo-G substrates, while it did not influence binding and cleavage of A:G substrates. On the other hand, a similar study, generating a 26 kDa N-terminal domain of MutY by trypsin-mediated proteolysis showed that this 26 kDa subunit was catalytically active, contained both DNA glycosylase and AP lyase activity, and was functionally identical with the full-length protein (Manuel et al., 1996; Manuel and Lloyd, 1997). A 14 kDa C-terminal domain of MutY (AA 1–226) was demonstrated to be the principal determinant for 8-oxo-G specificity, as its deletion remarkably enhanced the dissociation of the enzyme from A:8-oxo-G and reduced the rate of A removal from these substrates compared to A:G mismatches (Noll et al., 1999). This was interpreted such that the C-terminal domain facilitated A base flipping. Also, this study found that the C-terminal domain of MutY showed homology with MutT, suggesting that it might serve in 8-oxo-G recognition. Another report supported this view by showing that the N-terminal domain of MutY (AA 1–226) had a 18-fold lower affinity for binding various 8-oxo-G mismatches, a reduced catalytic preference for A:8-oxo-G over A:G mismatches and exhibited a lower inhibition on Fpg activity than the wild-type (wt) MutY (Li et al., 2000). These results suggested that the C-terminal domain of the protein determines its 8-oxo-G specificity and is crucial for mutation avoidance. The C-terminal domain was then shown to mediate additional contacts between MutY and A:8-oxo-G containing substrates that are not found in interaction with A:G (Li and Lu, 2000), thereby promoting the efficient recognition of substrates by MutY (Chmiel et al., 2001) and also affecting the catalytic activities toward A:G mismatches (Li and Lu, 2003). Taken together, the C-terminal domain of MutY seems to contribute substantially to the A:8-oxo-G substrate recognition.

It is still not entirely clear, how MutY is capable to efficiently recognize all its substrates from among the vast amount of undamaged base pairs. Along this line, the Fe-S cluster present in MutY was shown to be critical for the specific recognition of

its DNA substrate and its enzymatic activity (Porello et al., 1998a; Golinelli et al., 1999; Chepanoske et al., 2000a). It has also been suggested that the relative oxidation resistance of the Fe-S cluster may be an important aspect to guarantee the activity of MutY under conditions of oxidative stress (Messick et al., 2002). K142 in MutY, earlier shown to be involved in formation of tight interactions with DNA, was shown to make specific contacts with 8-oxo-G, and DNA-mediated charge transport (CT) was suggested as signal to promote the binding of MutY to DNA from a distance (Boon et al., 2002). Along this line, DNA-mediated CT led to oxidation of DNA-bound MutY, suggesting that G radicals provide the signal to stimulate DNA repair by the redox activation of DNA repair proteins through CT (Yavin et al., 2005). Further substantiating this idea, Boal et al. proposed that the rapid redistribution of proteins to the sites of DNA damage was mediated through redox activation involving the Fe-S clusters in proteins such as MutY and EndoIII (Boal et al., 2005; Yavin et al., 2006). A theoretical study of the DNA damage recognition by *Bacillus stearothermophilus* MutY proposed that the CT from MutY to DNA through hole transfer, which is specially efficient near an 8-oxo-G, leads to the stabilization of the enzyme in a conformation required for recognition of the lesion (Lin et al., 2008). Examination of the charge-transfer model by atomic force microscopy further validated this concept and emphasized the possibility that indeed repair proteins might be recruited to DNA lesions by DNA-mediated CT in the cellular context (Boal et al., 2009). The authors therefore proposed a model wherein the binding of Fe-S cluster containing DNA repair proteins (such as MutY and EndoIII) to DNA activates them toward oxidation. First, the formation of a guanine radical oxidizes a repair protein bound to DNA and thus stabilizes the binding of this protein. This step is followed by the binding of a second protein near the first one. Because also this protein gets oxidized during binding and transfers an electron to the DNA, it will induce a DNA-mediated CT from the second to the first protein if no damage is present in the DNA stretch between the two binding sites. This CT leads to reduction of the first protein and thus to its release from DNA, because in the reduced state it has a lower affinity to DNA. However, if there is a DNA lesion between the two bound proteins, the CT does not take place (it is “blocked” by the intervening lesion). In this situation both of the proteins remain bound and can subsequently catalyze repair steps. Through examination of CT mutants of EndoIII the group subsequently linked the ability of a repair protein to carry out DNA CT and its ability to localize to damaged DNA and thus further underlined their model (Romano et al., 2011). Taken together, the role for the Fe-S cluster as redox cofactor to search for damaged bases using DNA-mediated CT becomes more and more substantiated and really presents a plausible scenario to explain the mechanisms of full-genome search for lesions.

Base-excision. Investigations into the glycosylase activity of MutY revealed a distinctive difference in the processing of A:8-oxo-G compared to A:G mismatches (Porello et al., 1998b). Hydrolysis of A from opposite 8-oxo-G was at least 6-fold faster than from the A:G mispair. Interestingly however, MutY “lingered” when excising from an A:8-oxo-G base pair and released

the product with a much slower kinetic compared to the A:G substrate. This delay in substrate release might protect 8-oxo-G from being prematurely accessed and removed by other glycosylases, as also suggested by Zharkov and Grollman (1998). A detailed study of the active site revealed the importance of several amino acids involved in the glycosylase as well as DNA binding activities of MutY (Wright et al., 1999). Bifunctional glycosylases all bear a conserved lysine residue believed to be important for the initial nucleophilic attack in base removal near their active site, which is lacking in their monofunctional counterparts. To yield more insight into the role of this residue on a structural basis, Williams et al. investigated whether insertion of such a lysine residue into the catalytic site of MutY had any influence on its activity. Indeed, a point-mutation at S120K generated a MutY mutant capable of catalyzing DNA strand scission at a rate that was similar to its A excision activity from A:G and A:8-oxo-G substrates, and also changed it into a bifunctional glycosylase (Williams and David, 2000). This study illustrated that the basic mechanisms of mono- and bifunctional glycosylases were quite similar. The glycosylase activity of MutY was shown to involve a Schiff base intermediate, characteristic for other bifunctional DNA glycosylases that catalyze a β -lyase reaction, though no β -lyase step (*per se* only performed by bifunctional glycosylases) could be observed (Williams and David, 1998). Reduction of this Schiff-base intermediate with borohydride resulted in the formation of a covalent MutY-DNA adduct. To identify the residues involved in this covalent complex formation, Williams et al. constructed different MutY mutants and identified K142 to be the primary residue for such covalent associations (Williams and David, 1999). As the DNA binding and enzymatic activity of the K142A mutant was comparable to that of the wt enzyme, the formation of this covalent intermediate was not required for removal of A and was suggested to be a consequence of the unusually high affinity of MutY for the product of its glycosylase activity. Similarly, mutation of K142 to glutamine in MutY was shown to also abrogate its ability to form a Schiff base with DNA, while still retaining some of its catalytic activity (Zharkov et al., 2000). Interestingly, this mutation selectively impaired the processing of A:G base pairs, but not of A:8-oxo-G substrates, primarily by interfering with the binding to A:G substrates, but did not impair the catalytic activity *per se*, again confirming that it was not directly involved in the catalytic step. Using unnatural substrates to elucidate the tolerance of MutY to different modifications of the A or the 8-oxo-G in mismatches in an *E. coli*-based cellular assay, it was seen that, while modification of A was tolerated rather well, modification of 8-oxo-G resulted in a drastic reduction of base-excision (Livingston et al., 2008). This led to the conclusion that the presence of 8-oxo-G is critical for MutY to recognize A:8-oxo-G mismatches *in vivo* to initiate repair. D138 and Q37 are both residues that are involved in the catalytic mechanism of MutY-mediated A removal. Interestingly, their substitution yielded mutants with a range of different excision activities. Studies of these mutants demonstrated that changes which reduced the excision activity were better tolerated and less compromising to A:8-oxo-G repair *in vivo* in *E. coli* than those affecting the recognition of A:8-oxo-G mismatch affinity (Brinkmeyer et al., 2012). Therefore, this report suggested that the recognition of A:8-oxo-G mismatches was more important for the

correct repair of these duplexes than the actual glycosylase activity *per se*. Interestingly, this can be reconciled with the fact that the release of the substrate by MutY after base-excision is much slower than the actual N-glycosidic activity, seemingly demonstrating that the rate-limiting step of this enzyme is rather the identification of its substrate than the excision step itself. Additionally, this study also revealed which residues are critical for the selectivity and specificity of MutY.

Substrate release. The product release rate of MutY could be greatly enhanced by the two proteins AP-endonuclease IV and exonuclease III, and this effect depended on the presence of the C-terminal domain of MutY (Pope et al., 2002). Also, endonuclease VIII was found to promote MutY dissociation from AP:G substrates, but not from AP:8-oxo-G, and to further process these by $\beta\delta$ elimination (Lu et al., 2006b). This study also showed that MutY interacts with endo VIII through its C-terminus and competes with endo VIII on its natural substrate, the hydroxyurea

(hoU):A mismatch, thus inhibiting its activity and possibly reducing the mutagenic effects of hoU. Taken together, it seems important that also the substrate release step is tightly regulated, in order to orchestrate the following steps and to protect the 1-nt gap resulting from base-excision.

Structure of MutY and the removal of adenine opposite 8-oxo-G

The most precise structure of MutY comes from studies with *Bacillus stearothermophilus* (Lee and Verdine, 2009) (Figure 2). After binding to the 8-oxo-G:A mispair MutY flips out the A from the DNA double-helix. A water molecule is positioned between Asp144 and Asn146 in the MutY lesion-recognition pocket of the enzyme. Earlier studies included biochemical and computational studies on uracil DNA glycosylase (Werner and Stivers, 2000; Dinner et al., 2001) suggested that a so called dissociative action occurs, where the cleavage of the N-glycosidic bond and the subsequent attack of the water molecule on the C1' (arrow in Figure 2A) do not occur simultaneously, but rather in two

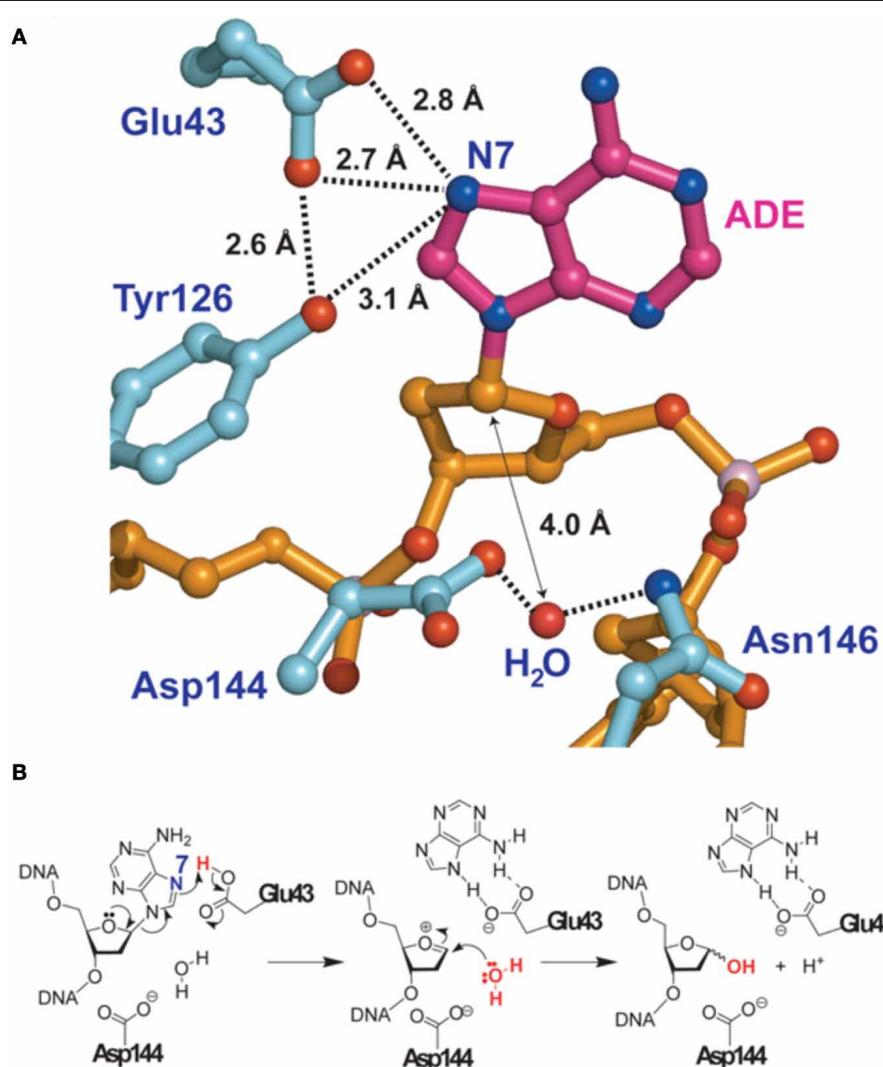


FIGURE 2 | Adenine removal by MUTYH. (A) View of the substrate adenosine interacting with catalytic residues of MUTYH. **(B)** Proposed glycolytic mechanism based on the structural information of **(A)**. Reproduced from Lee and Verdine (2009). For details see text.

discrete steps. In addition Glu43 can adopt a so-called bifurcated hydrogen-bonding interaction of 2.7 and 2.8 Ångströms, respectively, with N7 of A (**Figures 2A,B**). These short distances together with a protonated Glu43, provides acidity and therefore full hydrogen bonding to the N7 of A. As indicated in **Figure 2B** such a conformation favors the scission of the glycosylic bond between A and the deoxyribose. A similar structure has also been identified for human MUTYH, for which a fragment lacking the first 64 amino-acids was crystalized (Luncsford et al., 2010).

The structure of MutY catalytic core revealed that the two helical domains form a positively-charged groove, positioning the A-binding pocket at their interface (Guan et al., 1998). Also, this study confirmed a nucleotide flipping mechanism by a substitution of the Watson–Crick hydrogen bond partners by protein atoms. Recognition of 8-oxo-G seems to occur independently of double-stranded DNA or of an A-mismatch, and sequential extrusion of 8-oxo-G followed by A occurs in MutY, as demonstrated by Bernards et al. (2002). MutY has been proposed to assemble into a dimer upon substrate binding to yield an active form of the enzyme (Wong et al., 2003). This idea was further substantiated by a study that suggested a model for MutY binding of the mismatched DNA that involves scanning of the DNA by one molecule which enhances binding of second MutY molecule upon encountering an A:8-oxo-G mismatch (Lee et al., 2004).

Kinetically, it has been suggested that the release of A happens fast, while the rate-limiting step was the release of the AP-site (McCann and Berti, 2003). Further investigations into the transition state structure of MutY showed that the irreversible breakage of the N-glycosidic bond could not take place until a H₂O atom was present and that the enzyme stabilized the excision site after excision (McCann and Berti, 2008). Recently, a two-step reaction was proposed to be the basis of the catalytic activity of MutY, as opposed to the three-step mechanism proposed before (Tiwari et al., 2011).

Investigations of the roles of the different H₂O molecules involved in catalysis by MutY from *B. stearothermophilus* and *E. coli* suggested that E43 and N7 may be important factors for the activity of MutY (Brunk et al., 2012). Further insight into the roles of the substrate A residues N7 and N3 during catalytic excision by MutY have been gained recently (Michelson et al., 2012).

MutY in living cells

In *E. coli*, MutY was shown to be co-transcribed as first gene of a part of a large operon, together with Fpg, the bacterial DNA glycosylase which removes 8-oxo-G from the DNA (Gifford and Wallace, 1999). This further emphasized the involvement of MutY in the repair of 8-oxo-G base pairs in an interplay with Fpg and thus in the response to oxidative DNA damage. Somewhat surprisingly at first glance, oxidative stress down-regulated the activity of MutY by 70% as well as its mRNA levels, and in contrary it was induced more than 2-fold under anaerobic conditions (Yoon et al., 2003). This negative regulation of MutY was mediated by the regulatory genes fur, fnr and arcA. These results were explained with the idea that MutY activity had to be restrained when increased incorporation of 8-oxo-dGTP could possibly take place, which is during times of oxidative stress. This is important, because 8-oxo-dGTP could be inserted opposite a correct

templating A, which might erroneously get excised by the action of MutY, thus actually acting *promutagenic* in this scenario instead of protecting from mutations taking place.

Screening for mutator loci leading to GC→CG transversions in *E. coli*, Zhang et al. found that inactivation of MutY led to accumulation of these mutations (Zhang et al., 1998). As mentioned above, they showed that MutY bound to G:8-oxo-G mismatches and was capable of removing G from the G:8-oxo-G mispair.

To analyze the impact of *mutT*, *mutM* (which encodes the Fpg DNA glycosylase that removes 8-oxo-G from C:8-oxo-G base pairs in bacteria), and *mutY* on the mutational spectra, following considerations have to be taken into account. In the context of 8-oxo-G and 8-oxo-dGTP (1) CG→AT mutations can arise either from oxidation of C:G to C:8-oxo-G or from incorporation of 8-oxo-dGTP opposite C, followed by wrong incorporation of A opposite 8-oxo-G by the replicative pols during the next round of replication. (2) AT→CG mutations are based on incorporation of 8-oxo-dGTP opposite templating A. Analyzing different combinations of mutated strains in *mutT*, *mutY*, and *mutM*, Fowler et al. found that (1) *mutT* does not increase CG→AT transversions, regardless of the *mutY* and *mutM* background, suggesting that 8-oxo-dGTP does not often get incorporated opposite C but rather opposite A. (2) AT→CG transversions are reduced in *mutY* and *mutMmutY* backgrounds, suggesting templating 8-oxo-G preferentially pairs with dATP, which then is a substrate for MutY to excise A from the A:8-oxo-G pair, followed by Fpg that removes 8-oxo-G paired with C. And finally (3) *mutY* and *mutMmutY* decrease AT→CG mutations (arising from incorporation of 8-oxo-dGTP opposite templating A) in a *mutT wt* background, suggesting that a certain amount of 8-oxo-G gets incorporated into DNA even in the presence of functional MutT (Fowler et al., 2003). No strand bias in the mutation rate between leading and lagging strand synthesis in either a *mutMmutY* or a *mutT* background could be detected in *E. coli* using a supF shuttle vector (Watanabe et al., 2001). Interestingly, Bridges et al. showed that the rate of mutation markedly increased in starved *mutY* mutant *E. coli*, yielding CG→AT transversion mutations (Bridges et al., 1996). This phenotype could be further enhanced by additional mutation of *mutM*, even though mutation of *mutM* alone did not cause this effect. Also, addition of catalase to the plates did not alter the mutation rates, indicating that extracellular H₂O₂ was not involved in the generation of mutations, and it was suggested that singlet oxygen could be the source of internal DNA damage. These findings indicated that MutY may regulate the activity of Fpg in resting cells. Expression of MutY from a *mutY-lacZ* fusion construct was shown to be enhanced under aerobic compared to anaerobic conditions, but not to be down-regulated by nutrient limitation (Notley-McRobb et al., 2002). However, in many cases, nutrient limitation led to *mutY* inactivation by deletion, suggesting it might serve as a mechanism to increase mutation rates under these adverse conditions.

Clustered lesions, as induced by ionizing radiation, are defined as two or more lesions formed within one to two helical turns of the DNA. They present a challenge to the repair machinery of the cell. An 8-oxo-G in the vicinity of an AP site was found to retard the processing of the AP site by endo III and Fpg, and the AP site was found to elevate the mutation frequency at

8-oxo-G in *wt*, *nth*, *fpg*, and *mutY* deleted *E. coli* (Cunniffe et al., 2007). Interestingly though, the mutation frequency in *mutYfpg* null cells was reduced by the presence of the AP site, suggesting that the processing of tandem lesions challenges the cellular repair machineries. Similar findings by Noguchi et al. investigating the interplay of 1-nt-gaps and 8-oxo-G lesions in clusters in *E. coli* demonstrated again, that the mutagenic potential of 8-oxo-G depends on the presence and the position of the gap (Noguchi et al., 2012).

MutY competed with MutS-dependent mismatch-repair when A:C mispairs were present, especially in the presence of an increased dCTP pool (Kim et al., 2003). In *E. coli*, MutY has been shown to interact via its Fe-S cluster with the ATPase domain of MutS, which enhanced the binding affinity of MutY to A:8-oxo-G mismatches (Bai and Lu, 2007). MutY expression and activity were enhanced in a *mutS* mutant strain, and AT→GC transversions were reduced by additional mutation of *mutY* in a *mutS* background, suggesting a cooperative effect of MutY and MutS in repair of 8-oxo-G damage. Analysis of *Bacillus subtilis* revealed that the expression of MutY increased drastically upon deletion of *mutSL* operon in starved cells, possibly to disturb the balance between MutY and MMR proteins to support the production of mutations, which might give growth advantages to these cells (Debora et al., 2011).

In *Streptococcus mutans*, an oral pathogen, strains with mutations of *mutY* were shown to display elevated mutation rates, increased resistance to killing by acid and oxidative agents as well as higher virulence compared to the parent strain, suggesting that loss of a BER factor such as MutY could confer an advantage to pathogenic organisms (Gonzalez et al., 2012).

MutY and BER in *E. coli*

Reconstitution experiments with purified proteins from *E. coli* revealed, that the presence of Ape1, pol I, and DNA ligase is sufficient to catalyze the entire repair pathway of G:A mismatches *in vitro* (Au et al., 1989). Further elucidation of the pathway was achieved, when Radicella et al. showed that the average repair tract length initiated by MutY in *E. coli* is 9–27 nucleotides long, starting at the removed A, and involved pol I, even though the involvement of other pols was also evident (Radicella et al., 1993). This finding was further refined *in vitro*, when Tsai-Wu et al. found pol I to be responsible for generating these tracts of 5–12 nucleotides length (Tsai-Wu and Lu, 1994).

MUTYH

MUTYH activity and substrate specificity

The substrate specificities for MUTYH are summarized in **Table 1**. The mammalian homolog of MutY, MUTYH, was first purified from calf thymus and catalyzed removal of A from A:G, A:8-oxo-G and A:C mismatches (McGoldrick et al., 1995). Subsequently, expression and purification of the cloned human protein confirmed its activity to remove A from A:8-oxo-G and A:G base pairs *in vitro*, supporting that also the human homolog is a *bona fide* DNA glycosylase (Slupska et al., 1999). Purification of MUTYH from calf liver mitochondria yielded a protein that complexes with A:8-oxo-G, G:8-oxo-G, and T:8-oxo-G, weakly with C:8-oxo-G but not with A:G and A:C mismatches and

removed A mispaired with G, C, or 8-oxo-G while weakly removing G from G:8-oxo-G mispairs (Parker et al., 2000). Purification of the murine MUTYH revealed strong similarities to MutY function, even though the intrinsic rates of A removal were lower than by MutY (Pope and David, 2005). Shinmura and colleagues reported that both the purified nuclear and mitochondrial recombinant isoforms of human MUTYH were active, and predominantly removed A from A:8-oxo-G mispairs rather than A:G mispairs under physiological salt concentrations (Shinmura et al., 2000). MUTYH in human cell extracts was shown to be more active in binding and glycosylase activity toward A:G mismatches than recombinant MUTYH expressed in bacteria (Gu and Lu, 2001). Furthermore, the authors found this native form of MUTYH to migrate slower on a non-denaturing polyacrylamide gel than recombinant human MUTYH purified from bacteria. Moreover the native form seems to be phosphorylated, thus apparently enhancing its glycosylase activity predominantly on A:G but also on A:8-oxo-G. As the phosphorylation status of MUTYH did not alter its electric mobility, it was suggested to be possibly associated with other proteins to account for the higher apparent molecular weight. Accordingly, co-migration of APE1 and MUTYH with A:8-oxo-G substrates could be identified. Ohtsubo et al. found that MUTYH likely also harbors an activity to remove 2-OH-A (Ohtsubo et al., 2000). Removal of 2-OH-A from opposite 8-oxo-G or G has been described for murine MUTYH (Pope and David, 2005) and was confirmed for human MUTYH as well (Ushijima et al., 2005). MUTYH from *S. pombe* was able to remove G from G:8-oxo-G mismatches as efficiently as A from A:8-oxo-G mismatches, and its expression reduced the frequency of GC→CG transversions in an *E. coli* *mutY* mutant, suggesting it might be involved in the repair of G:8-oxo-G lesions (Doi et al., 2005).

A:8-oxo-G substrates processed by murine MUTYH were protected from inappropriate access by OGG1 and APE1, thus preventing the formation of DSBs (Tominaga et al., 2004).

A study by Miyako et al. found that mitochondrial DNA (mtDNA) from HeLa cells could be cleaved by recombinant *E. coli* MutY, in contrast to Fpg which has been shown to barely cleave mtDNA (Driggers et al., 1993; Hegler et al., 1993), and that this cleavage took place roughly at a rate that was expected to correspond to the amount of 8-oxo-G present in endogenous mtDNA (Miyako et al., 2000). Suzuki et al. investigated the repair of 8-oxo-G in DNA and 8-oxo-dGTP in 293T cells using supF shuttle plasmids (Suzuki et al., 2010). While knockdown of OGG1, MUTYH, NTH1, and NEIL1 all led to a significant increase in CG→AT transversions caused by the C:8-oxo-G pair in the shuttle plasmid, only knockdown of MUTYH resulted in a reduction in AT→CG transversions induced by 8-oxo-dGTP. In summary, MUTYH displays remarkable similarity to its bacterial homolog MutY regarding its activity and substrate specificity.

Localization of MUTYH

The subcellular localization of MUTYH was rather enigmatic for a long time. A study using expression of tagged proteins in COS-7 cells revealed that MUTYH was mainly transported to the mitochondria, which was probably the result of the isoform that was used (Takao et al., 1998). Follow-up work by the same group

identified an alternatively spliced transcript differing in exon 1, leading to the nuclear localization of this variant (Takao et al., 1999). Ten further isoforms containing unique 5' sequences that could be grouped into three types were subsequently described, and suggested to encode multiple authentic MUTYH proteins (Ohtsubo et al., 2000). Other reports have further discussed the localization of MUTYH in cells, finding isoforms targeted to the nucleus (Tsai-Wu et al., 2000; Ichinoe et al., 2004) or the mitochondria (Englander et al., 2002; Ichinoe et al., 2004). However, work still needs to be done to analyze the distribution of isoforms to the different subcellular compartments in different cell and tissue types to clarify this matter further.

Analyzing the distribution of endogenous MUTYH in serum-stimulated proliferating MRC5 cells with antibodies, Boldogh et al. reported both nuclear and mitochondrial localization of MUTYH (Boldogh et al., 2001). The nuclear form co-localized with BrdU and the proliferating cell nuclear antigen (PCNA) and, similarly to PCNA, increased 3- to 4-fold to peak during S-phase compared to G1, whereas levels of OGG1 or MTH1 did not change during the cell cycle. These studies suggested a role of targeting MUTYH to the replication fork to ensure that its activity is directed to the newly synthesized template strand. Subsequently, DNA replication was shown to enhance the MUTYH-dependent repair of A:8-oxo-G mismatches *in vivo*, and it was demonstrated that the interaction with PCNA was critical for the activity of MUTYH (Hayashi et al., 2002). Taken together, these findings clearly support a replication-associated function of MUTYH.

MUTYH and DNA damage signaling

Recently, a number of reports have accumulated that link MUTYH to the DNA damage response and implicate it in apoptotic signaling. To investigate the contribution of nuclear and mitochondrial accumulation of oxidative base lesions to the triggering of apoptosis, Oka and colleagues used *OGG1* knockout (ko) cells deficient in the nuclear or mitochondrial form of MUTYH, respectively (Oka et al., 2008). The accumulation of single-strand breaks in nuclear DNA triggered PARP-dependent cell death and could be rescued by depletion of nuclear MUTYH. The same was true for mitochondria, where MUTYH triggered calpain-dependent cell death by single-strand breaks. These results suggested that MUTYH catalyzes the formation of single-strand breaks in both of these DNAs, hence leading to the execution of apoptosis. Exposure of human cells to sodium nitroprusside, an agent that causes 8-oxo-G accumulation in cellular DNA by acting as an NO donor, led to MUTYH-dependent cell death that was initiated by oxidized bases in the mitochondrial, but not the nuclear DNA (Ichikawa et al., 2008). The role of single-strand breaks generated by MUTYH in the induction of cell death was further underlined by the finding that synthetic sickness/lethality mediated by either inhibition of pol β combined with MSH2, a component of the mismatch repair pathway, or pol γ with MLH1, both of which led to a nuclear 8-oxo-G accumulation, could be rescued by silencing of MUTYH (Martin et al., 2010). BER has been implicated in many different pathological conditions of the central nervous system (reviewed in Bosshard et al., 2012). A very recent report implicated MUTYH in degeneration by triggering apoptosis in microglia and neurons through initiation

of single-strand breaks during repair of A:8-oxo-G mismatches (Sheng et al., 2012). Nuclear accumulation of 8-oxo-G triggered PARP-dependent apoptosis in microglia, while mitochondrial 8-oxo-G accumulation led to calpain-dependent apoptosis in neurons. All these findings are in agreement with a model, wherein the repair of DNA mismatches by MUTYH leads to generation of toxic single-strand breaks, and thus contributes to cellular death in case of excessive damage burden (i.e., an amount of DNA damage that surpasses the cellular capacity to further process these lesions properly). Thus, this model explains, why under conditions of severe damage the *absence* of MUTYH is *beneficial for the survival* of the cells. On the other hand, there are a number of reports that show that *loss* of MUTYH actually can *sensitize* cells to DNA damaging agents. Along this line, double mutations in *S. pombe* *MUTYH* with *RAD1* or, to a lesser extent *RAD9*, were shown to enhance the sensitivity of the cells to DNA damaging agents and hydroxyurea (Jansson et al., 2008). The consequences of these deficiencies were chromosome segregation defects and checkpoint failure after UV irradiation, as well as morphological defects, even in the absence of DNA damaging agents. This implicated MUTYH in the repair of a wide range of DNA damage and linked it to the checkpoint pathway. Under low-dose oxidative stress, *MUTYH OGG1* double-ko mouse cells also showed hypersensitivity to oxidation damage and a reduction of S phase concomitant with an increase of G2/M phase cells, while the levels of cell death remained unchanged (Xie et al., 2008). Furthermore, an increase in centrosome amplifications and formation of multinucleated cells could be observed in the surviving fraction of the ko cells, suggesting an involvement of MUTYH and OGG1 in the regulation of cell-cycle progression and cell division under oxidative stress. Further evidence implicating MUTYH in checkpoint control came from a study showing that siRNA-mediated knockdown of MUTYH resulted in a decreased phosphorylation of ATR and Chk1 upon treatment of HEK293 cells with HU or UV (Hahm et al., 2011). Concomitantly, the authors observed an increase in the phosphorylation of Cdk2 as well as the amount of the Cdc25A phosphatase, suggesting that MUTYH was involved in activation of the DNA damage response.

Thus, there seems to be growing evidence that implicates MUTYH to be an important factor in the cellular response to oxidative stress and inflammatory conditions by an involvement in cell death signaling (as discussed in Oka and Nakabeppu, 2011). Along these lines, MUTYH has been suggested to play a role in mitochondrial dysfunction in the pathogenesis of Parkinson's disease (Fukae et al., 2007; Nakabeppu et al., 2007). However, it still remains to be clarified how MUTYH can initiate apoptosis of cells in some instances, while it seems to protect from apoptosis in others.

Impact of MUTYH knockout on oxidative DNA damage and tumorigenesis *in vivo*

The data on cells and mice with biallelic deletion of MUTYH are somewhat discrepant. MUTYH ko embryonic stem cells displayed a mutator phenotype, but did not show any hypersensitivity toward oxidative stress induced by H₂O₂ or menadione (Hirano et al., 2003). A study with *mutyh*^{-/-} knockout mice by Xie et al revealed no significant increase in survival or tumor incidence

after 14 months, suggesting that MUTYH deficiency is not sufficient to cause a tumor-predisposition (Xie et al., 2004). This study also showed that combined ko of MUTYH with OGG1 led to a decrease in life span and increased tumor formation for double ko mice compared to single ko. Interestingly, 75% of the lung tumors showed an activating GC→TA transversion mutation at codon 12 of K-ras, a feature that is often detected in MUTYH-associated polyposis (MAP) tumors, but none in the p53 gene or in the adjacent normal tissues. Additional heterozygosity for Msh2 (*mutyh*^{-/-} *ogg1*^{-/-} *msh2*[±]) did not inflict on the total tumor incidence but accelerated malignant lung and ovarian tumor formation in the *mutyh*^{-/-} *ogg1*^{-/-} background. A complete knockout of Msh2 to generate triple ko (*mutyh*^{-/-} *ogg1*^{-/-} *msh2*^{-/-}) further increased tumor incidence and decreased survival time, but did not differ from the phenotype displayed by *msh2*^{-/-} single knockouts. This was suggested to be due to the strong mutator phenotype of *msh2*^{-/-} mice that might mask additional difference caused by *mutyh*^{-/-} and *ogg1*^{-/-}.

Spontaneous mutagenesis in the small intestine of *ogg1*^{-/-} and *mutyh*^{-/-} *ogg1*^{-/-} double deficient mice at the age of 4–5 weeks using a transgene reporter revealed increased mutations in the double-ko's but not in the *ogg1*^{-/-} mice (Isogawa, 2004). Furthermore, the GC→TA mutation frequency increased in *mutyh*^{-/-} and in *ogg1*^{-/-} and a cooperative increase could be observed in *mutyh*^{-/-} *ogg1*^{-/-}, suggesting a cooperative function between OGG1 and MUTYH to prevent 8-oxoG-related mutagenesis. Russo et al. also reported an additive effect in *mutyh*^{-/-} *ogg1*^{-/-} on the age-dependent increase in 8-oxo-G levels in lung and small intestine compared to the single ko's (Russo et al., 2004). Strikingly, these tissues were identical with the ones that showed increased cancer incidence in *mutyh*^{-/-} *ogg1*^{-/-} mice in the study by Xie et al. (2004). MUTYH deficiency in a background of APC^{min/+} mice led to the occurrence of stop-codons in the APC gene by induction of CG→AT transversion mutations and thus promoted intestinal tumorigenesis (Sieber et al., 2004).

In 2007 a study reported an increased susceptibility to spontaneous and stress-induced tumorigenesis in a large cohort of *mutyh*^{-/-} mice kept for 18 months, strongly contradicting data on *mutyh*^{-/-} obtained by different groups thus far (Sakamoto et al., 2007). This suggested that presence of a MUTYH deficiency is sufficient to predispose for malignancies of the intestinal tract, such as lymphoma and adenoma. More impressively still, oral KBrO₃ treatment of *mutyh*^{-/-} mice led to a dramatic increase in CG→AT transversion mutations and small intestinal tumors. The authors claimed that the tumor-prone phenotype might have been missed earlier due to genetic differences in the mouse strains and the older age at which the tumor burden was evaluated in their study. This was in line with the fact that many of the studies with *mutyh*^{-/-} mice have been reporting a strong tendency toward age-dependent accumulation of 8-oxo-G in tissues. In general, in light of the huge complexity of the disease, it can be debated, whether mice are useful cancer models to compare with the human disease, due to the entirely different life span, metabolism, inbreeding status and many other aspects.

As noted above, the combination *mutyh*^{-/-} and *msh2*^{-/-} did not greatly affect the mutation rate. However, the loss of

mutyh^{-/-} combined with *msh2*^{-/-} significantly increased the amount of oxidative DNA damage in several organs compared to *msh2*^{-/-} mice, suggesting an independent contribution of both genes to genetic maintenance (Russo et al., 2009). Surprisingly, the development of metastasizing lymphoma and the time of death were significantly delayed in the *mutyh*^{-/-} *msh2*^{-/-} mice compared to *msh2*^{-/-}, suggesting that the cancer-prone phenotype of the double knockouts depends substantially on the activity of MUTYH (Russo et al., 2009). The relationship of MUTYH and MMR is reviewed in more detail in Russo et al. (2007).

In a mouse model of ulcerative colitis MUTYH was shown to play a major role in propagating the inflammatory response that lead to the onset of chronic colitis (Casorelli et al., 2010). Taken together, all the data analyzing the function of MUTYH *in vivo* strongly supports the idea that MUTYH-mediated correction of A:8-oxo-G mispairs plays an important role in the maintenance of genetic integrity and protects cells from malignant transformation.

THE MUTYH/POL λ BASE-EXCISION REPAIR PATHWAY

By catalyzing the excision of the mispaired A from A:8-oxo-G base pairs, MUTYH paves the way for a subsequent repair that ultimately reconstitutes an undamaged C:G base pair. MUTYH-initiated repair has been shown to involve a replication-coupled long-patch BER (LP-BER) pathway (Matsumoto, 2001; Parker et al., 2001; Yang et al., 2001; Parlanti et al., 2002). Along this line, a SP-BER pathway initiated by MUTYH was shown to be futile, because it uniquely generated A:8-oxo-G base pairs instead of the correct C:8-oxo-G base pairs, indicating that canonical MUTYH-initiated BER must proceed by the LP-BER sub-pathway (Hashimoto et al., 2004). For a long time it was unclear, which pol was capable of faithfully inserting a correct C opposite 8-oxo-G, as most examined pols showed significant error-prone bypass of 8-oxo-G (Shibutani et al., 1991; Pinz et al., 1995; Efrati et al., 1999; Prakash et al., 2000; Einolf and Guengerich, 2001; Vaisman and Woodgate, 2001; Krahn et al., 2003; Hsu et al., 2004). In 2007, our laboratory proposed that pol λ, together with its cofactors PCNA and replication protein A (RPA), inserts 1200-fold more efficiently the correct C opposite 8-oxo-G than the incorrect A (Maga et al., 2007). Furthermore, experiments with extracts from mouse embryonic fibroblasts (MEFs) deficient for pol λ suggested an important role of pol λ in bypass of 8-oxo-G. The importance of PCNA and RPA to determine the pol selection at 8-oxo-G lesions was further analyzed in a follow-up study. The two proteins were found to act as molecular switches to activate pol λ-dependent correct 8-oxo-G bypass and to repress the more error-prone pol β-dependent bypass (Maga et al., 2008). Subsequently, we showed that the MUTYH-initiated error-free LP-BER pathway involves pol λ (Maga et al., 2008; Van Loon and Hubscher, 2009), as depicted in detail in **Figure 1**. Herein, the monofunctional MUTYH excises the promutagenic A from A:8-oxo-G base pairs. This is followed by incision of the phosphodiester backbone 5' to the AP site by APE 1 that generates a 3'OH and a 5'dRP moiety, respectively. Thereafter, in the presence of RPA and PCNA, pol λ incorporates the correct C opposite 8-oxo-G and further elongates the primer by one more nucleotide (nt) downstream, thus generating a short 1-nt 5' flap. This overhang

is processed by flap endonuclease 1 (Fen1), resulting in a product that can be ligated by DNA ligase I. The resulting C:8-oxo-G base pair is then substrate for the canonical OGG1-initiated SP-BER as discussed above.

MUTYH-INTERACTING PROTEINS

All DNA damage repair pathways have to be tightly coordinated to ensure proper repair and to avoid the generation of cytotoxic and mutagenic intermediates. Protein-protein interactions either regulate the repair by recruitment of proteins to sites of DNA damage or modulate the catalytic activity of already bound enzymes.

MUTYH is interacting with proteins associated with the BER pathway, DNA replication and cell cycle checkpoints (**Table 2**). The first interaction partner of MUTYH was the endonuclease Ape1 (Parker et al., 2001; Yang et al., 2001). Ape1 stimulates the glycosylase activity of MUTYH independently from its own activity; a catalytically dead mutant of Ape1 still enhanced the cleavage efficiency of MUTYH on damaged DNA templates (Yang et al., 2001). Thus, the stabilization of the MUTYH-DNA complex was sufficient to enhance the repair capacity. Additionally, MUTYH and Ape1 were both recruited into a complex with A:8-oxo-G containing DNA in HeLa cell extracts (Gu and Lu, 2001). The interaction between the two proteins was suggested to be important to prevent the release of cytotoxic AP sites (Luncsford et al., 2010). MUTYH was found to interact with pol λ , as discussed above (Van Loon and Hubscher, 2009). Furthermore, the interaction of MUTYH with pol λ was enhanced by phosphorylation of pol λ by Cdk2/cyclinA (Markkanen et al., 2012b,c).

Gu et al identified the mismatch repair protein MSH6 as further interaction partner of MUTYH, and MSH6 regulated MUTYH by stimulating its glycosylase activity and binding capacity to A:8-oxo-G containing DNA (Gu et al., 2002).

MUTYH interacts with PCNA and RPA under conditions of unperturbed DNA replication. It was suggested that, upon encountering DNA damage, MUTYH switches to interact with the heterotrimeric ring-like molecule Rad 9, Rad1, and Hus 1, called the 9-1-1 complex (Parker et al., 2001; Shi et al., 2006).

Consistent with these findings, MUTYH co-localized with PCNA at replication foci in untreated cells (Boldogh et al., 2001). Also, replication was a prerequisite for MUTYH mediated repair to occur (Hayashi et al., 2002). The interaction site with PCNA was mapped to a conserved region within the MutY family, reflecting the importance of this interaction since PCNA directs MUTYH to the daughter strand where it excises a recently inserted mispaired A from A:8-oxo-G base pairs (Slupska et al., 1999). This directionality could also be the mechanism to make sure that MUTYH does not excise erroneously A from a base pair where 8-oxo-dGTP has been inserted opposite a templating (and thus correct) A. The interaction of MUTYH with PCNA and the structurally-related 9-1-1 complex was also confirmed in *S. pombe* (Parker et al., 2001; Chang and Lu, 2002, 2005; Shi et al., 2006; Luncsford et al., 2010). Interestingly, it was shown that even if the SpMUTYH does not have a perfect PCNA binding motif (Chang and Lu, 2005), cross-binding between the yeast and the human isoforms is possible and mutations within the PCNA binding domain impair the capability of MUTYH to repair A:8-oxo-G mismatches in yeast (Chang and Lu, 2002).

The 9-1-1 complex acts as a DNA damage sensor, blocks the cell cycle and simultaneously stimulates BER to allow repair to be completed before the DNA is replicated. The human MUTYH interacts with the h9-1-1 complex via binding to hRad1 and hHus1, but not to hRad9 (Shi et al., 2006). The glycosylase activity of MUTYH was stimulated by this interaction if 9-1-1 was present in a substantial molar excess. Treatment of cells with H₂O₂ or ionizing irradiation enhanced this interaction, supporting the hypothesis that 9-1-1 replaces PCNA in stress situations (Shi et al., 2006). Luncsford et al. identified the interdomain connector (IDC) of MUTYH to mediate the binding to 9-1-1 by providing a stabilized docking interface and proved the importance of the interaction by showing that mutations within this site decrease the repair of oxidative damage *in vivo* (Luncsford et al., 2010).

Partial interchangeability was observed between human and *S. pombe* homologs of these proteins, and enhanced glycosylase

Table 2 | Interaction partners of MUTYH.

Interaction partner	Species	Interaction site in MUTYH	Stimulatory effect
Ape1	human	259–318 (Parker et al., 2001)	Glycosylase activity (Yang et al., 2001)
MSH6	human	232–254 (Gu et al., 2002)	Glycosylase activity DNA binding (Gu et al., 2002)
Pol λ	human	Van Loon and Hubscher, 2009; Markkanen et al., 2012c 40–130 (Dorn et al., unpublished results)	n.d.
PCNA	human	505–527 (Parker et al., 2001), F518/F519 (Chang and Lu, 2002)	n.d.
	<i>S. pombe</i>	438–445 (Chang and Lu, 2002)	n.d.
9-1-1	human	295–350 (Shi et al., 2006) V315, E316 (Shi et al., 2006; Luncsford et al., 2010)	Glycosylase activity (Chang and Lu, 2005), interaction increased after IR (Shi et al., 2006)
	<i>S. pombe</i>	245–293 (Chang and Lu, 2005) I261, E262	Glycosylase activity (Chang and Lu, 2005), interaction increased after H ₂ O ₂ treatment
RPA	human	6–32 (Parker et al., 2001)	n.d.
ATR	human	n.d.	Checkpoint mediator? (Hahn et al., 2011)

n.d., not determined.

activity of *S. pombe* MUTYH was found with human Hus1 and the *S. pombe* 9-1-1. Human MUTYH was also observed to co-localize with Rad9 in cells treated with H₂O₂, suggesting that BER by MUTYH could be modulated by 9-1-1. Further work in *S. pombe* showed a decrease in repair of oxidative DNA damage *in vivo* when the interaction of MUTYH with 9-1-1 was disrupted, suggesting that this interplay significantly contributes to the response to oxidative stress (Luncsford et al., 2010). Also, MUTYH could be co-immunoprecipitated with ATR from human cells, possibly implicating MUTYH in ATR-mediated checkpoint execution (Hahn et al., 2011).

MUTYH from *S. pombe* was found to interact with Hst4, a histone deacetylase involved in silencing of genes and maintenance of genomic integrity, which seemed to regulate the levels of Hst4 after oxidative stress (Chang et al., 2011). Hst4 was further shown to interact also with the 9-1-1 complex. The association of MUTYH with telomeres was increased after oxidative stress and by deletion of Hst4, and Hst4 bound to telomeres decreased after oxidative stress, concomitant with a decrease in total Hst4 levels. Finally, MUTYH association with telomeres was increased in a Hst4 deletion background in the presence of chronic DNA damage caused by the lack of Hst4. Therefore, MUTYH seemed to regulate repair of telomeres by orchestrating the functions of 9-1-1 and Hst4. Finally, the WRN helicase/exonuclease was recently shown to promote MUTYH-initiated LP-BER of A:8-oxo-G mismatches by pol λ (Kanagaraj et al., 2012).

REGULATION OF MUTYH

REGULATION OF MUTYH LEVELS

So far, only a limited amount of studies has been performed concerning the regulation of MUTYH levels. Respiratory hypoxia caused a strong increase in mtDNA damage and also in expression of MUTYH mRNA in rat brain (Englander et al., 2002). This suggested that the increase denoted an adaptive mechanism for protection of neuronal DNA from oxidative injuries stemming from an imbalance in metabolism. Follow-up work by the same group identified specific MUTYH isoforms exclusive to brain tissue in rats, that were targeted to the mitochondria and some of them were inducible upon respiratory hypoxia (Englander et al., 2002). The divergence in the N-terminus between the different MUTYH isoforms was found to influence their excision rates and the processing of AP sites (Ma et al., 2004). In mononuclear blood cells MUTYH levels were neither altered by hypoxia nor by inhalation of 10% oxygen for 2 h and the subsequent reoxygenation period in healthy human subjects, even though DNA strand breaks and oxidatively damaged purines accumulated by this treatment (Risom et al., 2007). MUTYH, together with SMUG1, was regulated transcriptionally by p73, a member of the p53 protein family, through DNA damage induction by bile acid exposure, suggesting that this interplay regulates DNA damage repair (Zaika et al., 2011).

A comparison of embryonic stem cells to more differentiated cells did not reveal any impact on the mRNA levels of MUTYH, in contrast to OGG1, which decreased upon differentiation (Kuboyama et al., 2011). Alimentary supplementation with quercetin, a plant-derived flavonoid that has been attributed with anticarcinogen, was found to enhance the expression of MUTYH

in the distal colon mucosa of rats (Dihal et al., 2008). And finally, overexpression of hepatitis B virus X (HBx) was shown to increase 8-oxo-G levels in HepG2 cells, and to decrease the transcript levels of MUTYHα mRNA, while not affecting mRNA of OGG1, suggesting that this may be linked to the development of hepatocellular carcinoma which is associated to HBx infection (Cheng et al., 2009).

REGULATION OF MUTYH BY POSTTRANSLATIONAL MODIFICATIONS

Very little is known about the regulation of MUTYH by post-translational modifications (PTM) (Table 3). Findings from Gu et al. showed that MUTYH could be phosphorylated *in vitro* by different protein kinases (Gu and Lu, 2001). Comparison of the activity of native MUTYH from human cell extracts with in recombinant MUTYH purified from bacteria revealed a dramatic difference in the glycosylase activity, probably due to the phosphorylation state of the proteins. Indeed, the dephosphorylation of native MUTYH led to a tremendous loss of function on A:G or A:8-oxo-G mismatch containing templates. Differences in activity were also described for recombinant MUTYH expressed in bacteria or insect cells (Kundu et al., 2010). Mass spectrometric analysis confirmed S524 to be phosphorylated in the more active MUTYH, expressed in insect cells. Further functional studies using wt, phosphomimetic, or phosphodeficient mutants revealed an important role of S524 in substrate recognition and binding to DNA.

A defect in phosphorylation of MUTYH was also found to cause a mutator phenotype in different microsatellite stable colorectal cancer cell lines (Parker et al., 2002). All tested cell lines that showed elevated 8-oxo-G levels showed a decline in repair of A:8-oxo-G mismatches. While the sequencing of the MUTYH locus in these cells did not reveal any mutations, the mRNA and protein levels of MUTYH were decreased. In a subsequent study the same authors could demonstrate that a loss of MUTYH phosphorylation by PKC was responsible for the observed increase in 8-oxo-G causing the mutator phenotype (Parker et al., 2003). The 8-oxo-G repair capacity in MUTYH impaired cell extracts could be restored by complementation with PKC, PKA or casein kinase II. Furthermore, the same effect could be achieved by treatment with the PKC activator phorbol-12-myristate-13-acetate (PMA). In contrast to that, no effect in cell extracts from MUTYH proficient cells occurred, indicating that MUTYH was already

Table 3 | Posttranslational modifications of MUTYH.

Posttranslational modification	Site of modification	Kinase	Stimulatory effect
Phosphorylation (Gu and Lu, 2001)	n.d.	n.d.	Glycosylase activity
Phosphorylation (Parker et al., 2002, 2003)	n.d.	PKC PKA Casein Kinase II	Glycosylase activity
Phosphorylation (Kundu et al., 2010)	S524	n.d.	DNA-binding

n.d., not determined.

phosphorylated at a basal level in these cell lines. Consistent with these findings, MUTYH was a substrate for PKC *in vitro*. Finally, MUTYH purified directly from cell extracts treated with PMA showed an elevated capacity in the repair of A:8-oxo-G mismatches. So far it has not been elucidated whether phosphorylation only interferes with the catalytic activity of MUTYH, regulates its interaction with other proteins, or leads to a different subcellular localization. Since PKC can be stimulated by oxidative stress (Klein et al., 2000), it is possible that the phosphorylation-mediated regulation of MUTYH presents an adaptive response to DNA damage.

Taken together, it would be very interesting to investigate the regulation of MUTYH in more detail to get a better understanding how the different players of the 8-oxo-G repair machinery are controlled to protect cells from oxidative stress of endogenous or exogenous sources.

INVOLVEMENT OF MUTYH IN DISEASE

MAP (MUTYH ASSOCIATED POLYPOsis)

Familial adenomatous polyposis (FAP) is an autosomal dominant disease characterized by the formation of hundreds to thousands of adenomatous polyps in the colons and rectums of the affected patients (reviewed in Fearnhead et al., 2001). It is caused by a germline mutation in the adenomatous polyposis coli (*APC*) gene, mutations that are also responsible for 80% of the sporadic colorectal tumors. In 2002, Al-Tassan and co-workers studied a British family with multiple colorectal adenoma and carcinoma, but failed to detect a mutation in the *APC* gene (Al-Tassan et al., 2002). Closer analysis of the patient material revealed an increased tendency of somatic CG→AT transversion mutations in the *APC* gene, which is consistent with 8-oxo-G mediated mutagenesis. This observation led the authors to dissect the three enzymes that work synergistically to counteract 8-oxo-G mediated mutagenesis, namely OGG1, MUTYH, and MTH. Sequencing of the respective genes revealed two non-conservative mutations in the *MUTYH* gene, Y165C (through an A→G transition) and G382D (through a G→A transition), while no pathogenic changes were observed in the *OGG1* and *MTH* genes. The two mutations were found to reduce the activity of *E. coli* mutY to remove A from G:A mismatches by 98% and 86%, respectively, suggesting that a defect in MUTYH activity was the reason for the accumulated mutations in the patients and thus responsible for the APC-like phenotype. Subsequent work revealed that these mutations not only compromise the bacterial mutY, but also caused a decrease in the activity of human MUTYH for excision of A opposite 8-oxo-G, which nicely correlated with the tumor phenotype (Al-Tassan et al., 2002; Chmiel et al., 2003; Pope and David, 2005). Further investigation led to the identification of seven other unrelated patients with colorectal adenomas or carcinomas that showed a bias of CG→AT transversion mutations to be carriers of biallelic germline mutations for MUTYH (Jones et al., 2002). This disorder is the only colorectal cancer form inherited in an autosomal recessive mode and is now commonly referred to as MAP, or infrequently also as FAP2 (<http://www.omim.org>). The prevalence of MAP is estimated to be around 1% of all colorectal cancer cases (Enholm et al., 2003; Croitoru et al., 2004; Fleischmann et al., 2004; Wang et al., 2004; Peterlongo et al., 2005; Webb et al., 2006;

Kury et al., 2007; Cleary et al., 2009) and MUTYH mutations have been found in 7% (Filipe et al., 2009), and 10% (Pezzi et al., 2009) of FAP patients and 40% of AFAP patients, respectively (Filipe et al., 2009). The lifetime-cancer risk is assessed to 80% for colon cancer and 4% for duodenal cancer (Jasperson et al., 2010). Even though MAP is a rather recently discovered disease, many germline mutations in addition to the two found by Al-Tassan et al have been described so far. This is reflected in the abundance of literature investigating different single-nucleotide polymorphisms and their relevance to cancer development has been thoroughly reviewed in Cheadle and Sampson (2007) and Poulsen and Bisgaard (2008). Interestingly, other than MUTYH, no association of further genes involved in BER or the repair of oxidative DNA damage with a multiple colorectal adenoma phenotype has been found so far (Dallosso et al., 2008). Interestingly though, work by the Sweasy group has found that the *POLB* gene is mutated in many colorectal cancers, suggesting that at least some of these mutations may lead to compromised BER function in the affected tissues (Donigan et al., 2012; Nemec et al., 2012). MAP patients have been reported to be more prone also to extraintestinal tumors such as ovarian, bladder, skin, and breast cancer. For an overview of all extracolonic manifestations of MAP-patients, please refer to this recent review (Nielsen et al., 2011). For further clinical features, there are excellent recent reviews available (Jasperson et al., 2010; Nielsen et al., 2011). Several mutations in MUTYH associated with MAP were found to significantly enhance the spontaneous mutator phenotype of patient's lymphoblasts under conditions of oxidative stress and to accumulate 8-oxo-G in the DNA, underlining the role of MUTYH in the pathogenesis of this disease (Ruggieri et al., 2012). However, for many of the mutants it is unclear how the mutation affects its activity, and more work is needed to clarify their exact contribution to the disease.

EQUINE CEREBELLAR ABIOTROPHY

Interestingly, MUTYH has been suggested to be involved in the pathogenesis of equine cerebellar abiotrophy, a neurological disease found in Arabian horses, as indicated by a SNP in the GATA2 binding region of the *MUTYH* promoter (Brault et al., 2011). Whether there is a real causative role and what mechanisms are behind it, remains to be elucidated by further studies.

CONCLUSIONS AND PERSPECTIVES

The MUTYH DNA glycosylase is a remarkable enzyme since it has the specificity to remove an undamaged DNA base from a mismatch such as an A:8-oxo-G. It is found throughout evolution from bacteria to human, suggesting an essential role in preventing mutations arising from oxidative damage to the DNA. During the last three decades, our knowledge about how MUTYH functions has grown substantially. We now understand quite in detail how MUTYH acts catalytically, and the structures of prokaryotic and eukaryotic enzymes have been identified. However, the functional details of the at least 10 isoforms of MUTYH, are far from being unequivocally clarified. MUTYH acts together with pol λ in the so-called MUTYH/pol λ pathway that can replace a promutagenic A paired to an 8-oxo-G with a correct C. The interaction with the moving platforms PCNA and the 9-1-1

complex is apparently very important for the proper spatial and temporal engagement of MUTYH on the DNA, and there especially in the context of chromatin. So far, very little is known about the regulation of MUTYH, which is at least in part likely achieved by PTM. Phosphorylation as an important PTM contributes to regulate the activity of MUTYH. It is likely that other PTM's, such as ubiquitination, will be identified that govern the temporal (i.e., during the cell cycle) as well as the spatial (i.e., the subcellular localization) distribution of MUTYH. Also, the fact that mutations in MUTYH are identified in diseases of human and animals shifts this enzyme more and more into the focus of translational medicine. In the future, it will be of interest to understand more

about the subcellular localization and specific functions of the different isoforms of MUTYH. Also, the exact regulation of the activity, stability, and localization of this enzyme is likely to yield many novel insights. Finally, we are anticipating further clarification of the functional roles of the different mutations in MUTYH associated with MAP.

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Controlling DNA-end resection: a new task for CDKs

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DNA double-strand breaks (DSBs) are repaired by two major pathways: homologous recombination (HR) and non-homologous end-joining (NHEJ). The choice between HR and NHEJ is highly regulated during the cell cycle. DNA-end resection, an evolutionarily conserved process that generates long stretches of single-stranded DNA, plays a critical role in pathway choice, as it commits cells to HR, while, at the same time, suppressing NHEJ. As erroneous DSB repair is a major source of genomic instability-driven tumorigenesis, DNA-end resection factors, and in particular their regulation by post-translational modifications, have become the subject of extensive research over the past few years. Recent work has implicated phosphorylation at S/T-P motifs by cyclin-dependent kinases (CDKs) as a major regulatory mechanism of DSB repair. Intriguingly, CDK activity was found to be critically important for the coordinated and timely execution of DNA-end resection, and key players in this process were subsequently identified as CDK substrates. In this mini review, we provide an overview of the current understanding of how the DNA-end resection machinery in yeast and human cells is controlled by CDK-mediated phosphorylation.

Keywords: DNA double-strand break repair, DNA-end resection, homologous recombination, cyclin-dependent kinase, phosphorylation, CtIP/Sae2, PIN1

INTRODUCTION

In order to preserve genome integrity, cells employ a complex surveillance network that detects, signals and repairs DNA lesions. These intricate and highly regulated pathways are collectively termed the DNA damage response (DDR; Zhou and Elledge, 2000). One major hallmark of the DDR represents the activation of checkpoints to temporarily delay cell cycle progression through inhibition of cyclin-dependent kinase (CDK) activity. In the budding yeast *Saccharomyces cerevisiae*, a single CDK, Cdc28 (or Cdk1), drives both G1/S and G2/M transitions, whereas in metazoan four CDKs are responsible for cell cycle progression (Morgan, 1997). CDK activity is modulated by association with regulatory subunits known as cyclins, the levels of which oscillate during the cell cycle (King et al., 1996). G1 phase is controlled by CDK4 and CDK6 in complex with D-type cyclins, whereas CDK2-cyclin E is essential for G1/S transition and the assembly of the DNA replication machinery. CDK2-cyclin A is required for proper completion of DNA replication and progression through S phase. Toward the end of interphase, cyclin A associates with CDK1 to facilitate S/G2 transition before CDK1-cyclin B complexes drive cells through mitosis (Morgan, 1997; Malumbres and Barbacid, 2005). CDKs belong to a large family of proline-directed kinases (which also includes MAPKs and GSK3) that exclusively phosphorylate serines or threonines immediately preceding a proline (S/T-P motifs) (Hanks and Hunter, 1995; Errico et al., 2010). CDK substrate specificity is increased by direct binding of the cyclin subunit to conserved RxL motifs present in certain CDK targets (Harper and Adams, 2001). A recent study showed that 50% of CDK2-cyclin A targets carried at least one RxL motif distal to the phosphorylation site (Chi et al., 2008).

In accordance with reduced CDK activity as a consequence of DNA damage-induced checkpoint activation, S/T-P motifs are largely dephosphorylated in response to DNA double-strand breaks (DSBs) (Bennetzen et al., 2010; Beli et al., 2012). However, in apparent contrast to this, CDK activity is strictly required for accurate processing and repair of DSBs in S/G2 phase, indicating that at least some DDR factors are primed by CDK phosphorylation prior to checkpoint activation (Enserink and Kolodner, 2010; Chapman et al., 2012). DSBs are highly deleterious lesions with the potential to cause cell death or genomic instability leading to cancer. DSBs can arise spontaneously as a result of replication fork collapse or can be induced by exogenous DNA-damaging agents including ionizing radiation and certain anti-cancer drugs (Jackson and Bartek, 2009). In order to repair DSBs, all organisms rely on two major pathways: non-homologous end-joining (NHEJ) and homologous recombination (HR). NHEJ functions throughout the cell cycle and religates broken ends without the need of extensive processing (Lieber, 2010). HR, instead, requires an undamaged template for faithful DSB repair, usually the sister chromatid, and is therefore restricted to S/G2 phase (Heyer et al., 2010). HR is initiated by 5'-3' degradation of the DSB ends to generate 3'-single-stranded DNA (ssDNA) overhangs. This evolutionarily conserved process, termed DNA-end resection, requires the coordinated action of several nucleases and helicases (Figure 1; Mimitou and Symington, 2009; Blackwood et al., 2013). Recent work in yeast and human cells has established that DNA recombination and particularly DNA-end resection are highly regulated by various kinases including Mec1/ATR, Tel1/ATM, Rad53/CHK1, Cdc5/PLK1, and, as reviewed here, CDKs (Longhese et al., 2010; Chapman et al., 2012; Finn et al., 2012; Krejci et al., 2012).

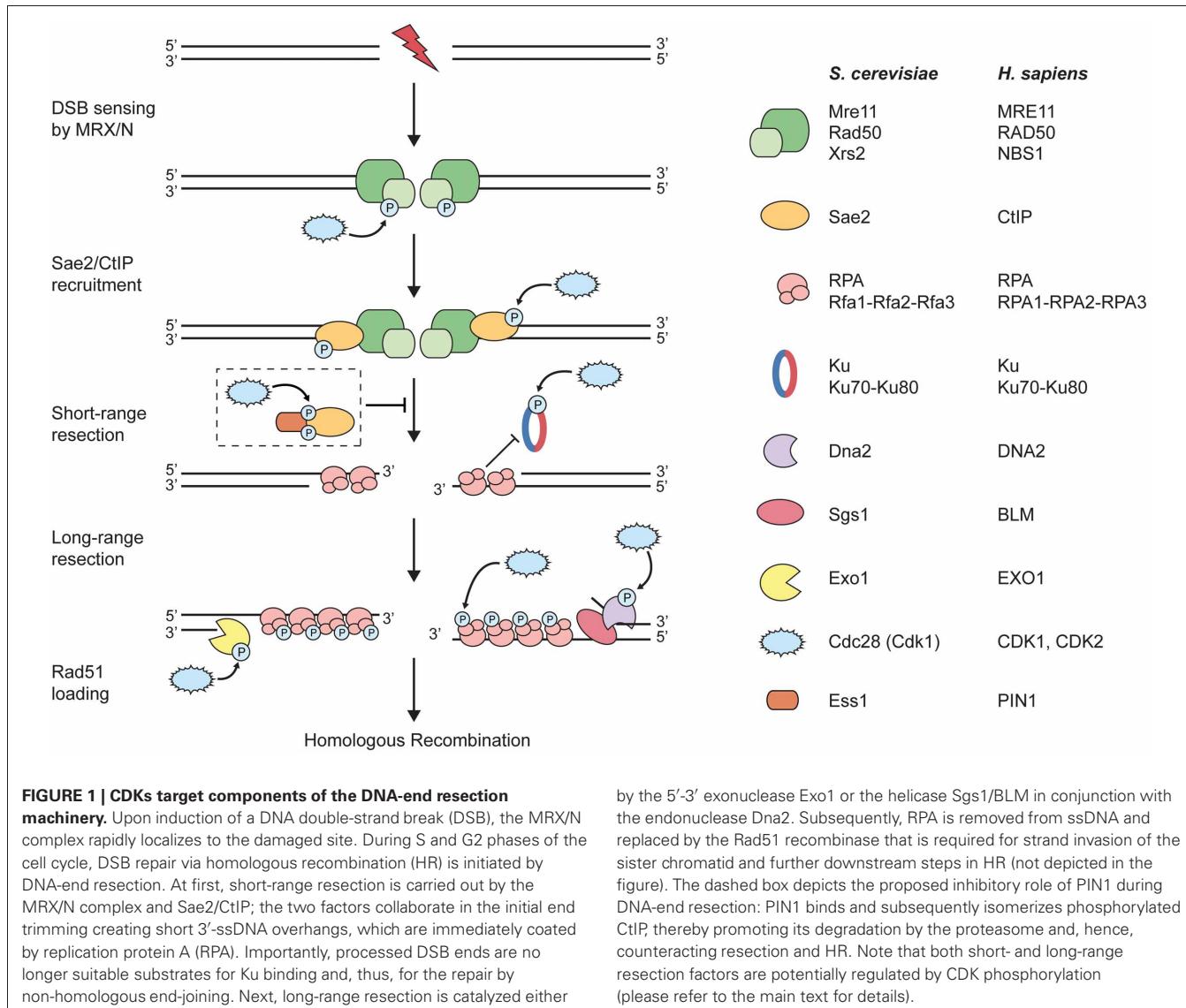


FIGURE 1 | CDKs target components of the DNA-end resection machinery. Upon induction of a DNA double-strand break (DSB), the MRX/N complex rapidly localizes to the damaged site. During S and G2 phases of the cell cycle, DSB repair via homologous recombination (HR) is initiated by DNA-end resection. At first, short-range resection is carried out by the MRX/N complex and Sae2/CtIP; the two factors collaborate in the initial end trimming creating short 3'-ssDNA overhangs, which are immediately coated by replication protein A (RPA). Importantly, processed DSB ends are no longer suitable substrates for Ku binding and, thus, for the repair by non-homologous end-joining. Next, long-range resection is catalyzed either

by the 5'-3' exonuclease Exo1 or the helicase Sgs1/BLM in conjunction with the endonuclease Dna2. Subsequently, RPA is removed from ssDNA and replaced by the Rad51 recombinase that is required for strand invasion of the sister chromatid and further downstream steps in HR (not depicted in the figure). The dashed box depicts the proposed inhibitory role of PIN1 during DNA-end resection: PIN1 binds and subsequently isomerizes phosphorylated CtIP, thereby promoting its degradation by the proteasome and, hence, counteracting resection and HR. Note that both short- and long-range resection factors are potentially regulated by CDK phosphorylation (please refer to the main text for details).

CDK SUBSTRATES IN DNA-END RESECTION

In 2004, two studies in *S. cerevisiae* described for the first time that Cdk1 is essential for DSB repair pathway choice by promoting DNA-end resection in G2 phase (Aylon et al., 2004; Ira et al., 2004). These findings were later confirmed in human cells, showing that ssDNA-dependent activation of the ATR checkpoint pathway in response to DSBs is restricted to S/G2 and requires CDK activity (Jazayeri et al., 2006). Similarly, inhibition of CDK2 in mammalian cells was shown to impair HR and delay DSB signaling (Deans et al., 2006). Based on these key findings, it was proposed that DNA-end resection is governed by CDK-mediated phosphorylation (Figure 1) (Ira et al., 2004). However, it was only until the last few years that components of the resection machinery were identified as CDK substrates.

MRX/MRN

Genetic studies in *S. cerevisiae* have long implicated the Mre11-Rad50-Xrs2 (MRX) complex in the initial processing of DSBs

(Symington and Gautier, 2011). However, as MRX exhibits both endonuclease and 3'-5' exonuclease activities *in vitro* (Paull, 2010), it still remains unclear how MRX catalyzes 5'-3' nucleolytic degradation of DNA ends *in vivo*. New clues came from a recent study suggesting that DNA-end resection could occur with bidirectional polarity, as opposed to the unidirectional model shown in Figure 1. Accordingly, Mre11 endonuclease first creates a nick in the strand to be resected up to 300 nucleotides away from the DSB that, in a second step, serves as an entry point for resection by Mre11 3'-5' exonuclease toward the DSB end and by Exo1 5'-3' exonuclease away from the DSB (Garcia et al., 2011).

None of the MRX subunits have so far been reported as Cdk1 substrates. Moreover, an *mre11* mutant in which all six S/T-P motifs have been mutagenized did not exhibit any major phenotypes attributable to a resection defect. The same holds true for an *xrs2* mutant in which both CDK consensus motifs (S/T-P-x-K/R) were mutated (Ira et al., 2004). Notably, however, three additional S/T-P motifs in Xrs2 were found to be phosphorylated

in a proteomic study, raising the possibility of it being indeed a Cdk1 substrate (Albuquerque et al., 2008). In human cells, akin to the situation in yeast, only the NBS1 subunit of the MRN complex was found to be phosphorylated in a cell-cycle-dependent manner (Figure 1; Olsen et al., 2010). Additionally, two groups reported that CDKs phosphorylate NBS1 at serine 432 in S phase (Falck et al., 2012; Wohlbold et al., 2012). Surprisingly, while Falck et al. concluded that NBS1-S432 phosphorylation promotes DNA-end resection, Wohlbold et al. reported normal resection in the absence of NBS1-S432 phosphorylation. Although it is rather difficult to reconcile these contradicting results, they have most likely emanated from the different NBS1-deficient cells used for complementation studies. Thus, it remains to be clarified whether Xrs2/NBS1 phosphorylation by CDKs is a conserved mechanism to promote DNA-end resection by MRX/N.

Sae2/CtIP

SAE2 (or COM1) was originally identified as being required to complete meiotic recombination in *S. cerevisiae* (McKee and Kleckner, 1997; Prinz et al., 1997). Subsequent genetic and biochemical studies in yeast and mammalian cells have shown that Sae2 and its human counterpart CtIP cooperates with the MRX/N nuclease to initiate resection of DSBs (Figure 1; Sartori et al., 2007; Symington and Gautier, 2011). There are three potential CDK phosphorylation sites in Sae2 and 12 in CtIP. Remarkably, phosphorylation of a single S/T-P motif in the C-terminus of both proteins (Sae2-S267/CtIP-T847) by CDK is required to promote resection (Huertas et al., 2008; Huertas and Jackson, 2009). Consistent with a role of Cdk1 in positively regulating Sae2 function, mutation of a RxL cyclin-binding motif present upstream of S267 caused comparable DNA damage hypersensitivity to that of *sae2*-S267A cells (Huertas et al., 2008). Moreover, in cells expressing a phospho-mimicking mutant (Sae2-S267E/CtIP-T847E), resection is permitted even in absence of Cdk1 activity; however, not to the same extent as in normal cells. Therefore, it was proposed that additional Cdk1 sites, on Sae2/CtIP itself or on other proteins, are required for optimal resection (Huertas, 2010). Despite the fact that the precise mechanism of how S267/T847 phosphorylation “activates” Sae2/CtIP is still unclear, it is of major importance for both meiotic and mitotic recombination (Manfrini et al., 2010; Nicolette et al., 2010).

Prior to the identification of CtIP-T847 as a CDK site, phosphorylation of S327 was shown to occur exclusively during S/G2 and to be a pre-requisite for CtIP-BRCA1 interaction (Yu and Chen, 2004; Yu et al., 2006). Furthermore, it was recently shown that CtIP-S327 phosphorylation is CDK2-dependent and facilitated by MRE11, which directly interacts with CDK2 and CtIP, thereby bringing CDK2 in proximity with its substrate (Buis et al., 2012). Although evidence for a direct role of CtIP-S327 phosphorylation in resection is still missing, the BRCA1-CtIP complex was recently reported to facilitate the removal of the 53BP1 effector protein RIF1 from DSBs in S/G2, thereby channeling DSB repair into HR (Escribano-Díaz et al., 2013). Moreover, it was recently reported that phosphorylation of a cluster of five additional S/T-P motifs located in the central region of CtIP is important for DNA-end resection (Wang et al., 2013). Mechanistically,

phosphorylation of this cluster is needed for the association of CtIP with NBS1, which promotes DNA damage-induced CtIP phosphorylation by ATM (You et al., 2009; Wang et al., 2013). It is important to note, however, that Wang et al. did not directly address whether any of these clustered phosphosites in CtIP are indeed targeted by CDKs *in vivo*.

KU

When DSBs arise in the cell, Ku—a heterodimer composed of Ku70 and Ku80—is usually loaded onto duplex DNA ends. During the repair process, Ku serves as a docking site for many NHEJ proteins, including DNA-PKcs and DNA ligase IV, to rejoin the broken ends (Lieber, 2010). It has been shown that DNA-end resection and HR are constrained during G1 due to both efficient NHEJ and low CDK activity (Aylon et al., 2004; Jazayeri et al., 2006). Interestingly, in the absence of Ku, Cdk1 activity is dispensable for the initiation of resection by MRX-Sae2, but is still needed for long-range resection by Exo1 or Sgs1-Dna2 (Clerici et al., 2008). Therefore, Ku is thought to antagonize DNA-end resection and has to be removed from the ends in order to permit HR. These data also indicate that CDK activity promotes resection by restraining the recruitment of Ku to DSBs, raising the question whether Ku itself is a potential CDK substrate (Figure 1). However, removal of all putative Cdk1 phosphorylation sites on Ku70 and 3 out of 4 sites on Ku80 failed to elicit any DSB repair phenotype in *S. cerevisiae*, suggesting that the negative regulation of Ku by Cdk1 is most likely indirect (Zhang et al., 2009). Ku binding to DNA ends also attenuates resection and HR in mammalian cells (Shao et al., 2012; Tomimatsu et al., 2012). Furthermore, Ku70 was reported as a binding partner and substrate of CDK2-cyclin A, and Ku70-T455 was identified as a CDK target site by mass spectrometry (Müller-Tidow et al., 2004; Chi et al., 2008; Olsen et al., 2010); but whether or not Ku phosphorylation by CDKs has an impact on DNA-end resection has yet to be determined.

EXO1

Exonuclease 1 (Exo1) belongs to the RAD2/XPG family of structure-specific 5' nucleases and has been implicated in multiple genome maintenance pathways including DNA repair and telomere maintenance (Tran et al., 2004). Exo1 is dispensable for initial resection in yeast and human cells but acts in a separate pathway from Sgs1-Dna2/BLM-DNA2 to promote extensive 5'-3' DSB resection (Figure 1; Symington and Gautier, 2011). Moreover, Exo1-dependent resection and its recruitment to DSBs depends on both MRX/N and Sae2/CtIP and is blocked by the presence of Ku (Eid et al., 2010; Sun et al., 2012; Tomimatsu et al., 2012). Although DNA damage-induced phosphorylation of Exo1 has been reported to attenuate its activity in both yeast and human cells (Morin et al., 2008; Bolderson et al., 2010), probably by controlling its stability (El-Shemery et al., 2005), there is currently no published data available whether Exo1 is a CDK target. However, several S/T-P sites in human EXO1 were repeatedly found to be phosphorylated using mass spectrometry analyses (El-Shemery et al., 2008; Chen et al., 2009; Shiromizu et al., 2013). Indeed, some of these sites are phosphorylated by CDKs in S/G2 phase, thereby stimulating DNA-end resection by EXO1 and

promoting DSB repair by HR while at the same time suppressing NHEJ (S. Burma, personal communication).

Sgs1-Dna2/BLM-DNA2

Sgs1 and its human ortholog BLM are members of the RecQ family of 3'-5' DNA helicases and are involved in the suppression of crossovers by promoting the dissolution of Holliday junction intermediates (Bernstein et al., 2010). The role for Sgs1 in conjunction with the Dna2 nuclease in the generation of long stretches of ssDNA during HR was discovered because of its redundancy with Exo1 (**Figure 1**; Gravel et al., 2008; Mimitou and Symington, 2008; Zhu et al., 2008). Although there is currently no data available on CDK-mediated phosphorylation of Sgs1, BLM is phosphorylated at various S/T-P motifs by mitotic kinases including CDK1 (Beausoleil et al., 2004; Leng et al., 2006; Dephoure et al., 2008; Olsen et al., 2010). However, these modifications are more likely to be involved in the regulation of BLM's function in the separation of sister chromatids during mitosis rather than in DNA-end resection (Chan and Hickson, 2011). In contrast, Cdk1-mediated phosphorylation of *S. cerevisiae* Dna2 at T4, S17, and S237 stimulates its recruitment to DSBs and DNA-end resection (Chen et al., 2011). Consistent with the redundancy observed between Dna2- and Exo1-dependent resection pathways, *dna2-T4A/S17A/S237A* cells only resect DSBs in the presence of functional Exo1. Interestingly, T4 and S17 lie within a bipartite nuclear localization signal, suggesting a timely regulated nuclear import of Dna2 upon phosphorylation during G1/S transition (Kosugi et al., 2009). Remarkably, human DNA2 lacks the entire N-terminal region of yeast Dna2 including all three S/T-P sites, suggesting that CDK-mediated regulation of long-range resection in human cells differs from yeast.

RPA

Replication protein A (RPA) is an evolutionarily conserved, heterotrimeric complex consisting of RPA1, RPA2, and RPA3. Owing to its high ssDNA binding affinity, RPA is required for most aspects of DNA metabolism including replication, repair and recombination (Oakley and Patrick, 2010). Following resection, RPA wraps around the generated 3'-ssDNA overhangs to protect the DNA against nuclease degradation and to prevent hairpin formation that would impede Rad51 filament assembly (**Figure 1**; Holloman, 2011). *In vitro* studies have also implicated RPA in promoting long-range resection through stimulation of both Exo1- and Sgs1-Dna2-dependent pathways (Cejka et al., 2010; Niu et al., 2010; Nimonkar et al., 2011; Cannavo et al., 2013). Furthermore, under DNA-damaging conditions, RPA-coated ssDNA serves to recruit the Mec1/ATR kinase, a critical event in checkpoint activation (Zou and Elledge, 2003). RPA2 contains a flexible N-terminal domain that is differentially phosphorylated at multiple residues during the cell cycle and in response to genotoxic stress. Two residues within this region, S23 and S29, are phosphorylated by CDK2-cyclin A and CDK1-cyclin B at the G1/S boundary and during mitosis, respectively (**Figure 1**); however, they are not conserved in yeast (Oakley and Patrick, 2010). In response to DSBs, ATR-mediated phosphorylation of RPA2-S33 induces phosphorylation of RPA2-S23/S29, and both act synergistically to stimulate phosphorylation of additional

residues closer to the N-terminus by DNA-PK (Anantha et al., 2007; Liaw et al., 2011). Although DNA damage-induced RPA2 hyper-phosphorylation seems critical for Rad51 recruitment and HR in response to replication stress, it is not essential for HR as measured by an I-SceI-based reporter assay (Shi et al., 2010; Serrano et al., 2013). Moreover, dephosphorylation of RPA2 by the PP4 phosphatase complex has also been reported to facilitate HR (Lee et al., 2010). However, a direct role of CDK-mediated RPA phosphorylation in DNA-end resection has not yet been demonstrated.

CHROMATIN BINDING AND REMODELLING FACTORS

DNA-end resection occurs in the context of chromatin, which constitutes a natural barrier to all kind of DNA transactions including DSB repair (Price and D'Andrea, 2013; Tsabar and Haber, 2013). Last year, three groups described a role of the *S. cerevisiae* chromatin-remodeling factor Fun30 (and its human counterpart SMARCAD1) in the repair of DSBs by HR (Chen et al., 2012; Costelloe et al., 2012; Eapen et al., 2012). Fun30/SMARCAD1 physically associates with DSB ends and, by weakening the histone-DNA interactions in nucleosomes, establishes a DNA conformation that facilitates both Sgs1- and Exo1-dependent resection. Furthermore, it was shown that Fun30 function in resection becomes less important in cells lacking the histone-bound Rad9 checkpoint protein, suggesting that Fun30 helps to overcome the inhibitory effect of Rad9 on DNA-end resection (Chen et al., 2012). Interestingly, both Fun30 and Rad9 were identified as Cdk1 substrates and reported to be phosphorylated at multiple S/T-P sites (Ubersax et al., 2003; Albuquerque et al., 2008). Moreover, loss of Rad9 has been reported to partially bypass the requirement for Cdk1 in resection (Lazzaro et al., 2008). This inhibitory mechanism is likely to be evolutionarily conserved as 53BP1, the mammalian ortholog of Rad9 (Wang et al., 2002), suppresses resection to promote NHEJ and immunoglobulin class switching (Bunting et al., 2010; Bothmer et al., 2011). Accordingly, multiple CDK consensus sites in SMARCAD1 and 53BP1 were repeatedly found to be phosphorylated (Beausoleil et al., 2004; Linding et al., 2007; Bennetzen et al., 2010; Olsen et al., 2010; Shiromizu et al., 2013). Further experiments are required to establish whether some of the CDK sites in Fun30/SMARCAD1 and Rad9/53BP1 play a role in the regulation of DNA-end resection and, thus, in DSB repair pathway choice.

CONCLUDING REMARKS

While the role of CDKs in regulating DNA-end resection is a given fact, we are only beginning to understand the mechanistic consequences of these phosphorylation events for individual repair factors, e.g., on protein-protein interactions, intracellular localization, or protein stability. Another important question to address in the future is how DNA-end resection is limited in order to generate confined tracts of ssDNA that are suitable for homology search by the Rad51 recombinase leading to productive HR. In other words, there must be additional regulatory mechanisms providing a switch between activation and inhibition of DNA-end resection to coordinate DSB repair pathways in a spatiotemporal manner.

Novel insights are provided by a recent study showing that PIN1, a phosphorylation-specific peptidyl-prolyl cis/trans isomerase, counteracts DNA-end resection in human cells (Steger et al., 2013). PIN1 was previously shown to isomerize phosphorylated S/T-P peptide bonds, thereby controlling the function of a subset of CDK substrates involved in diverse cellular processes (Liou et al., 2011). In a proteomic screen for PIN1 substrates, Steger et al. identified several prominent DSB repair proteins including BRCA1, 53BP1 and CtIP. Interestingly, PIN1-mediated isomerization of CtIP requires the phosphorylation of CtIP at two S/T-P sites: CtIP-pT315 (by CDK) serves as the major binding site for PIN1, whereas CtIP-pS276 (by an unknown proline-directed kinase) is isomerized by PIN1. Following isomerization, CtIP gets ubiquitylated and subsequently degraded by the proteasome. In this way, PIN1 is proposed to limit DNA-end resection, thereby possibly contributing to fine-tune the coordination of HR and NHEJ during S and G2 phases of the cell cycle (Figure 1; Karanam et al., 2012). So far, no direct connection has been made between

PIN1 and the regulation of DSB repair in *S. cerevisiae*, studies of which are hampered by the fact that yeast PIN1 (Ess1) is essential for viability (Siepe and Jentsch, 2009). Future studies will have to determine whether phosphorylation-dependent regulation by PIN1 in concert with CDKs applies to other DSB repair proteins apart from CtIP and, thus, represents a general feature of the DDR.

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Put a RING on it: regulation and inhibition of RNF8 and RNF168 RING finger E3 ligases at DNA damage sites

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RING (Really Interesting New Gene) domain-containing E3 ubiquitin ligases comprise a large family of enzymes that in combination with an E2 ubiquitin-conjugating enzyme, modify target proteins by attaching ubiquitin moieties. A number of RING E3s play an essential role in the cellular response to DNA damage highlighting a crucial contribution for ubiquitin-mediated signaling to the genome surveillance pathway. Among the RING E3s, RNF8 and RNF168 play a critical role in the response to double stranded breaks, one of the most deleterious types of DNA damage. These proteins act as positive regulators of the signaling cascade that initiates at DNA lesions. Inactivation of these enzymes is sufficient to severely impair the ability of cells to respond to DNA damage. Given their central role in the pathway, several layers of regulation act at this nodal signaling point. Here we will summarize current knowledge on the roles of RNF8 and RNF168 in maintaining genome integrity with particular emphasis on recent insights into the multiple layers of regulation that act on these enzymes to fine-tune the cellular response to DNA lesions.

Keywords: RING E3 ligase, genomic stability, RNF8, RNF168, DNA damage response, ubiquitin, NHEJ, HR

INTRODUCTION

Eukaryotic cells have evolved sophisticated mechanisms to detect and repair different types of DNA lesions, collectively referred to as the DNA damage response (DDR). In response to DNA lesions this pathway marks the damaged DNA, activates cell cycle checkpoints that halt cellular proliferation and activates the DNA damage repair machinery. Double strand DNA breaks are among the most deleterious types of DNA lesions and, strikingly, the presence of only a few DNA damage sites is sufficient to fully engage the DDR pathway resulting in a robust cell cycle inhibition (Bakkenist and Kastan, 2003). Several steps of regulation must ensure that the damage signal does not spread beyond the site of lesion and that its activation is reversed only upon completion of the DNA repair events. We now appreciate that an efficient response to DNA damage involves transcriptional regulation, micro-RNA biogenesis, detection of methylated histone tails, and multiple posttranslational modification events including phosphorylation, sumoylation, and ubiquitylation.

In this review, we will focus on the role of ubiquitin signaling in the response to double stranded breaks (DSBs). In recent years, an expanding view of ubiquitylation as a means to modulate the timing and efficiency of the repair process and to recruit factors to the sites of DNA lesions has emerged. Ubiquitin (Ub) is an essential 76-amino-acid protein conserved from yeast to humans. Ubiquitylation is a three-step enzymatic process through which ubiquitin becomes covalently attached to specific target proteins. First, ubiquitin is activated by an E1 activating enzyme via an ATP-dependent reaction to form an E1-Ub thioester bond. Then, the activated ubiquitin is transferred to an E2 conjugating enzyme. Finally, an E3 ligase catalyzes the transfer of ubiquitin to a target

protein through formation of an isopeptide bond between the carboxyl-terminus of ubiquitin and a lysine (K) residue on the target protein (Ciechanover et al., 1982; Hershko et al., 1983). It is the E3 enzyme that confers the majority of the substrate specificity to the ubiquitylation cascade through recognition of a distinct set of target proteins.

The two major classes of E3 ligases are the RING (really interesting new gene)/Ubox domain-containing E3s and the HECT (homologous to E6-associated protein C-terminus) domain-containing E3s. While HECT E3s form an intermediate thioester bond with ubiquitin, RING E3s act as scaffolds, facilitating ubiquitylation by bringing the E2 and substrate close together.

RING finger ubiquitin ligases comprise one of the largest families of enzymes in human cells with more than 600 members (Deshaias and Joazeiro, 2009). They are the most abundant class of E3s and regulate many crucial cellular functions, such as cell cycle progression, DDR, DNA repair, cell signaling, and response to hypoxia (reviewed in Lipkowitz and Weissman, 2011). RING E3s catalyze target monoubiquitylation or polyubiquitylation assembled via different lysine residues of ubiquitin, which have a range of different biological effects, from proteasomal degradation (K48-linked polyubiquitylation) to regulation of DNA repair, receptor internalization, and gene silencing, among others (monoubiquitylation and/or K63-linked polyubiquitylation; Johnson, 2002; Sun and Chen, 2004).

Highlighting the relevance for ubiquitin signaling in organism homeostasis, mutations in RING E3s are frequently associated with human diseases such as BRCA1 (breast cancer type 1 susceptibility protein) mutations in patients with breast and ovarian cancer (Hashizume et al., 2001; Ruffner et al., 2001), and MDM2

(mouse double minute 2 homolog) amplification in several human cancers (Oliner et al., 1992; Wade et al., 2010).

Multiple RING E3s play a central role in various DDR pathways and are involved in either sensing or repairing DNA lesions, some of which are listed in **Table 1**.

In this review, we will focus primarily on RNF8 (RING finger 8) and RNF168 (RING finger 168), which are essential in the cellular response to DSBs and appear to form a key nodal point of regulation to modulate the DSB response and repair pathway.

We will also describe recently reported cross-talks between the RNF8/RNF168 pathway and other cellular components: (i) RING E3s of the polycomb group (PcG) complex PRC1 (polycomb repressive complex 1), which are emerging as potential novel players in the response to DSBs, where they appear to primarily contribute to the transcriptional silencing that occurs at sites of DNA lesions; (ii) proteins involved in small ubiquitin-like modifier (SUMO) signaling, whose role in DSB repair and response is becoming increasingly evident.

RNF8/RNF168: MAJOR PLAYERS IN THE DETECTION OF DNA DOUBLE STRAND BREAKS

The response to DSBs is initiated by the phosphatidylinositol 3-kinase-related kinase ATM (ataxia-telangiectasia mutated), which rapidly accumulates at DNA lesions in a MRN (MRE11/RAD50/NBS1)-dependent manner (Bekker-Jensen et al., 2006). ATM activation results in the phosphorylation of the histone variant H2AX on serine 139 (referred to as γ -H2AX). This marks the nucleosomes surrounding the DNA lesion and serves as an anchoring platform for the subsequent accumulation of downstream signaling proteins to DSB sites (Rogakou et al., 1998; Burma et al., 2001). MDC1 (mediator of DNA damage checkpoint 1) binds directly to γ -H2AX via its BRCT domains (Stucki et al., 2005). MDC1 recruitment results in amplification of the DNA damage signal by promoting further accumulation of the MRN complex at DSBs (Chapman and Jackson, 2008; Melander et al., 2008; Spycher et al., 2008). RNF8 and RNF168 are recruited at this step in the DDR pathway in an MDC1-dependent manner (**Figure 1**). RNF8 localizes to DSBs via its N-terminal forkhead-associated (FHA) domain, which interacts with the ATM-phosphorylated TQXF motifs on MDC1 (Huen et al., 2007; Kolas et al., 2007; Mailand et al., 2007). In concert with

the E2 enzyme UBC13, the ligase activity of the C-terminal RNF8 RING finger domain is responsible for the recruitment of BRCA1 and 53BP1 (p53 binding protein 1; see below for more details; Wang and Elledge, 2007).

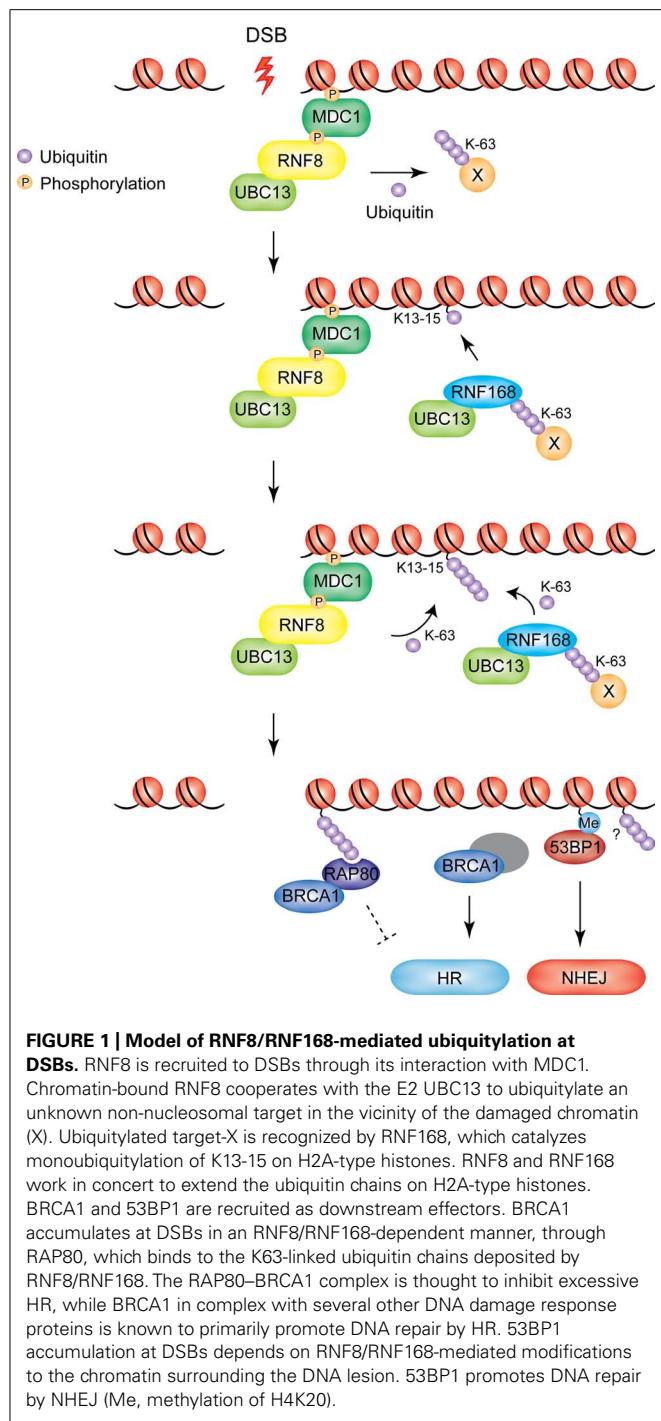
Historically, evidence has suggested that the targets of RNF8-UBC13 at DSBs are the histones H2A and H2AX, as they were shown to be ubiquitylated in response to DSBs in an RNF8-dependent manner (ubiquitylated H2A, uH2A; Huen et al., 2007; Mailand et al., 2007). Depletion of RNF8 leads to an impaired G2/M checkpoint at low doses of ionizing radiation (IR) and hyper-sensitivity to IR, indicating that the RNF8-dependent signaling is critical for the cellular response to DNA damage (Huen et al., 2007; Kolas et al., 2007; Mailand et al., 2007; Wang and Elledge, 2007).

RNF168 was identified in genome-wide screens as a factor necessary for IR-induced 53BP1 focus formation (Doil et al., 2009; Stewart et al., 2009). Significantly, mutations of RNF168 are associated with the RIDDLE syndrome (radiosensitivity immunodeficiency dysmorphic features and learning difficulties), which is characterized by cellular defects in repairing DSBs. Cells derived from RIDDLE patients fail to recruit 53BP1 and BRCA1 at irradiation-induced DNA damage sites (IRIFs) and can be complemented by exogenous RNF168 expression (Stewart et al., 2009). RNF168 is a RING-type ubiquitin ligase that also works with the UBC13 E2 enzyme. The RING domain of RNF168 is not required for recruitment at DNA lesions, which is instead dependent on its two ubiquitin-binding motifs MIU1 and MIU2 (motif interacting with ubiquitin; Doil et al., 2009; Pinato et al., 2009; Stewart et al., 2009; Panier et al., 2012). However, the catalytic activity of RNF168 is required for the accumulation of 53BP1 and BRCA1 at sites of damage. RNF168 activity is also required for the accumulation of IR-induced uH2A, an activity that can be recapitulated *in vitro* (Doil et al., 2009; Stewart et al., 2009). RNF168 recruitment at damage sites is RNF8-dependent, while RNF8 is recruited to DSBs in a RNF168-independent manner (Doil et al., 2009; Stewart et al., 2009). These data supported the widely accepted model for the sequential recruitment of RNF8 and RNF168 at sites of DNA damage, whereby RNF8 would initiate ubiquitin conjugation on histones H2A and H2AX. Subsequently, RNF168 would be recruited at damaged sites through the binding of its MIU domains to uH2A, resulting in further amplification of the ubiquitin signal

Table 1 | RING finger E3s involved in DDR pathways.

RING E3	Target	Ubiquitylation type	Function
RNF8	H2A/H2AX and other unknown	K63 chains	DSB signaling and repair
RNF168	H2A/H2AX	Mono on K13-15 of H2A/H2AX and K63 chains	DSB signaling and repair
BRCA1	Unknown	K6 and other unknown?	Promote HR
BMI1	H2A (H2AX?)	Mono on K119 of H2A	Gene silencing; DSB signaling?
RING1B	H2A (H2AX?)	Mono on K119 of H2A	Gene silencing; DSB signaling?
RAD18	PCNA	Mono on K164 of PCNA	PRR
FANCL	FANCD2/FANCI	Mono on K561 of FANCD2, on K523 of FANCI	ICL repair (FA pathway)

Mono, monoubiquitylation; *PRR*, post-replication repair; *ICL*, inter-strand crosslink; *FA*, Fanconi anemia.



via the formation of K63-linked polyubiquitin chains (Panier and Durocher, 2009). This model has been recently challenged by the discovery that RNF8 is inactive toward nucleosomal H2A (Mat-tioli et al., 2012). This study provides evidence that the initial ubiquitylation of histone H2A is mediated by RNF168 at K13-15. The authors propose a model in which RNF8 is responsible for the ubiquitylation of other non-nucleosomal proteins localized at the DNA damage site, which would represent the docking site for RNF168. Thus, recruitment of RNF168 in this model is still

dependent on RNF8 but does not involve ubiquitylation of nucleosomal H2A (or H2AX) as the priming step. RNF168 catalyses the monoubiquitylation of H2A and H2AX at K13-15. Subsequently, K63-linked ubiquitin chains can be conjugated by both E3 ligases in concert (Figure 1). This model is also in keeping with a recent report from the Durocher laboratory, which suggests the existence of two waves of RNF168 recruitment to DSBs: an initial transient recognition of RNF8-dependent ubiquitylation, followed by a more stable association to DSB-flanking chromatin promoted by RNF168 catalytic activity itself (Panier et al., 2012).

DNA damage-induced non-proteolytic polyubiquitin chains catalyzed by RNF8 and RNF168 in DSB-flanking chromatin serve as binding sites for the recruitment of the downstream effectors of the DDR pathway BRCA1 and 53BP1 (Figure 1, bottom panel) (Kolas et al., 2007; Mailand et al., 2007; Wang and Elledge, 2007; Doil et al., 2009). The relative dynamics with which these two components accumulate at break sites is extremely important to determine the choice of repair pathway the cell will take to ensure genome stability, underscoring the central role played by RNF8/RNF168 in orchestrating the DDR and repair pathways.

In mammalian cells, DSBs are predominantly repaired by the homologous recombination (HR) and the non-homologous end-joining (NHEJ) pathways. NHEJ is the primary repair mechanism during G0-, G1-, and early S-phases of the cell cycle (Delacote and Lopez, 2008). The NHEJ process ligates the broken DNA molecule back together and, due to the varying levels of end processing prior to end-joining, this pathway is often error-prone (reviewed in Lieber, 2008). Conversely, the HR pathway is an error-free repair process that utilizes the sister chromatid as a template to repair damaged DNA and is thus only active in the S/G2 phase of the cell cycle (Moynahan et al., 1999; Wyman and Kanaar, 2006). 53BP1 and BRCA1 have reciprocal roles in DSB repair: BRCA1 is required for efficient HR, while 53BP1 promotes NHEJ (see Figure 1; Nakamura et al., 2006; Xie et al., 2007; Difilippantonio et al., 2008; Dimitrova et al., 2008; Yun and Hiom, 2009). Several recent reports have shown how the antagonism between these two proteins is important for DSB repair pathway choice and consequent cell survival. A striking example of this antagonism is provided by cells that have impaired BRCA1 activity: inhibition of 53BP1 in this background is able to restore viability and suppress genomic instability associated with defective BRCA1, by allowing resection of DSBs and repair via the HR pathway to occur (Bouwman et al., 2010; Bunting et al., 2010). Below we will summarize current knowledge on how RNF8 and RNF168-dependent signaling recruits these important factors to sites of DSBs.

MECHANISMS OF RNF8/RNF168-DEPENDENT RECRUITMENT OF DOWNSTREAM EFFECTOR PROTEINS

53BP1

Recruitment of 53BP1 to damaged chromatin was shown to occur via recognition of dimethylated histone H4 on K20 (H4K20me2) by its tandem Tudor domain (Sanders et al., 2004; Botuyan et al., 2006). 53BP1 does not contain any known ubiquitin-binding motifs and therefore the mechanism through which RNF8/RNF168 mediates its recruitment is still not fully understood. Recent findings from different groups point to a model in which RNF8/RNF168-dependent ubiquitylation at DSBs is

necessary to remove factors that bind to H4K20me2 sites, thus unmasking the sites for 53BP1 binding. Meerang et al. (2011) demonstrated that RNF8 is responsible for the recruitment of the AAA ATPase p97/VCP to the sites of DNA damage via both K63- and K48-linked ubiquitin chains. p97/VCP was shown to be in turn responsible for the removal of the polycomb protein L3MBTL1 from H4K20me2 histones at damage sites (Acs et al., 2011). These findings suggest that removal of L3MBTL1 is necessary for 53BP1 binding to H4K20me2, although this hypothesis remains to be fully validated. In another study by Mallette et al. (2012), RNF8/RNF168-dependent unmasking of H4K20me2 sites was indeed found to be involved in 53BP1 recruitment to DSBs. In their work, the authors show that the Tudor domain-containing lysine demethylases JMJD2A and JMJD2B, which bind to H4K20me2, become polyubiquitylated and degraded in response to DNA damage and that this requires the E3 ligase activity of both RNF8 and RNF168. The combined knockdown of JMJD2A and JMJD2B significantly rescued the ability of RNF8- and RNF168-deficient cells to form 53BP1 foci upon DNA damage induction, indicating that the RNF8/RNF168-dependent degradation of the lysine demethylases controls the recruitment of 53BP1 at DNA damage sites. Although these findings indicate that removal of H4K20me2-binding proteins is certainly important for the recruitment of 53BP1 to DSBs, it is likely that other mechanisms exist which could explain the requirement for H2AK13-K15 ubiquitylation in this process (Mattioli et al., 2012). An additional mechanism that may contribute to 53BP1 DSB recruitment is the DNA damage-induced chromatin relaxation that occurs at sites of DNA lesions, which may allow H4K20me2 to be more accessible for 53BP1 binding (Xu et al., 2010; Luijsterburg and van Attikum, 2012). A more detailed description of this process will be provided in Section “Chromatin Remodeling” of this review.

BRCA1

BRCA1 is a RING E3 ubiquitin ligase and is recruited to DNA as a dimer with BARD1 (BRCA1-associated RING domain 1), which enhances BRCA1 RING domain ligase activity *in vitro*. BRCA1 has multiple roles in the DDR and repair pathways, underscored by its recruitment to damaged DNA in several different complexes. These complexes have functions in sensing DNA damage, controlling cell cycle checkpoints, and recruiting DNA repair enzymes to orchestrate the cross-talk of the DNA repair pathways with various cellular processes (Huen et al., 2010). It is generally accepted that the main function of BRCA1 in DNA repair is to promote HR (reviewed in Yun and Hiom, 2009; Caestecker and Van de Walle, 2013). At DSBs, BRCA1 is recruited to RNF8/RNF168-generated ubiquitin chains as part of the BRCA1-A complex [containing BRCA1, BARD1, RAP80 (receptor-associated protein 80), ABRAXAS, BRCC36, BRE, and NBA1] through RAP80, which binds to the K63-linked ubiquitin chains deposited by RNF8/RNF168 at DNA lesions via its two N-terminal UIMs (ubiquitin interacting motif; Kim et al., 2007; Sobhian et al., 2007; Wang and Elledge, 2007; Wang et al., 2007). The exact role of the BRCA1-A complex is not fully understood; although RAP80- and ABRAXAS-depleted cells display mild defects in HR (Wang et al., 2007), recent reports have shown that the BRCA1-RAP80 complex can inhibit HR by restricting end-resection (Coleman

and Greenberg, 2011; Hu et al., 2011). Further work is required to establish the exact role of the different BRCA1 complexes in the repair of DSBs.

MODULATION OF THE RNF8/168 PATHWAY

The choice of the DNA repair mechanism is crucial for cell survival after genotoxic insult. The relative accumulation at DSBs of 53BP1 and BRCA1, which determines this choice, is dependent on RNF8/RNF168, underscoring the necessity of a highly stringent regulation mechanism for these enzymes. In the following sections, we will illustrate the various means by which mammalian cells are able to achieve multiple layers of regulation in order to tightly control RNF8/RNF168 signaling, with particular focus on recent reports in the field. We will also describe how regulation of RNF8/RNF168 is exploited in different settings: by the telomere protection machinery to safeguard the integrity of chromosome ends, and by viruses during infection of mammalian cells.

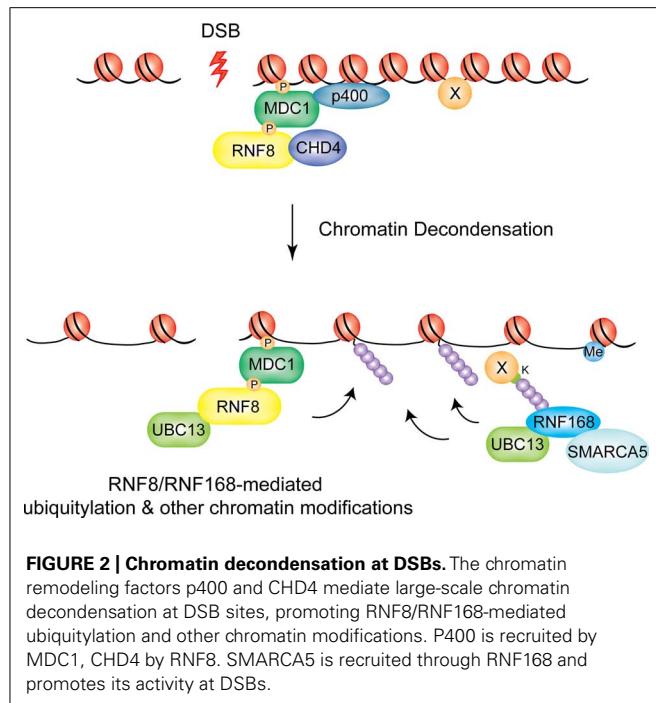
CHROMATIN REMODELING

Chromatin remodeling plays an important role in the DDR pathway to create a local chromatin environment that facilitates the assembly of checkpoint response and repair factors (reviewed in Luijsterburg and van Attikum, 2012; Soria et al., 2012). Indeed, several chromatin remodeling factors are recruited to sites of DNA damage and have been shown to modulate the activity of RNF8 and RNF168 at sites of damage.

p400, the catalytic subunit of the NuA4 histone acetyltransferase complex, is recruited at DSBs in a MDC1-dependent manner (Xu et al., 2010). Interestingly, in the absence of p400, while RNF8 is still recruited to DSBs, polyubiquitin chains are not formed at the sites of damage. As a consequence, BRCA1 and 53BP1 recruitment is impaired and p400-deficient cells show radiosensitivity and chromosomal aberrations. The mechanism through which this occurs is still unclear, but the authors speculate that chromatin relaxation may alter the nucleosome structure to expose previously buried histone domains, which can then be targeted by RNF8. In the light of the recent report from the Sixma group described above, one possibility is that p400-mediated chromatin relaxation is an important step to expose lysine residues on a so far unidentified RNF8 target (see model in **Figure 2**). This nucleosome destabilization was also suggested to contribute to exposure of methylated histone residues for binding of 53BP1.

A similar function seems to be played by CHD4 (chromodomain helicase DNA binding protein 4) of the NuRD histone deacetylase complex. CHD4 is recruited to laser-induced DNA damage sites in a RNF8-dependent manner, where it promotes efficient RNF8-mediated ubiquitin conjugation and recruitment of downstream factors RNF168 and BRCA1, presumably by favoring chromatin decondensation (Luijsterburg et al., 2012). Interestingly, RNF8 interaction with CHD4 involves the FHA domain of RNF8 while it does not require E3 catalytic activity.

Finally, SMARCA5, an ATPase contained in several distinct ISWI chromatin remodeling complexes has been shown to have a role in RNF8/RNF168-dependent DDR signaling (Smeenk et al., 2012). Smeenk and colleagues show that SMARCA5 is recruited to DSBs induced by laser micro-irradiation and its depletion increases IR-sensitivity and impairs both the HR and NHEJ repair



pathways. SMARCA5 and RNF168 interact at DSBs in a mutually dependent fashion that requires the catalytic activity of PARP1. Surprisingly, SMARCA5-depleted cells do not display any defect in 53BP1 accumulation at IIRFs, even though ubiquitin conjugation and BRCA1 assembly were significantly reduced. This implies that either the threshold for ubiquitin levels required to promote BRCA1 or 53BP1 recruitment differs, or that alternative mechanisms of 53BP1 recruitment exist when the SMARCA5-RNF168 response is impaired. Further studies will be required to establish how SMARCA5 and the other chromatin remodeling enzymes described above remodel chromatin in the vicinity of DSBs to promote the RNF8/RNF168 signaling cascade, thereby promoting genomic stability in response to genotoxic insult.

Collectively, these data suggest a “chromatin remodeling-assisted ubiquitylation” activity for RNF8 and RNF168. In this model p400 and CHD4 are required to promote the initial ubiquitylation events at DNA damage sites that are needed to recruit RNF168, which, in turn, propagates the ubiquitylation signal and promotes accumulation of downstream effectors. In addition, SMARCA5 plays a role at the level of RNF168 promoting its activity following the initial ubiquitylation event (see model in **Figure 2**).

DE-UBIQUITYLATION ACTIVITIES AT SITES OF DNA DAMAGE

Ubiquitin-mediated signaling at sites of DNA damage can be attenuated or turned off by the action of de-ubiquitylating enzymes (DUBs; Sowa et al., 2009).

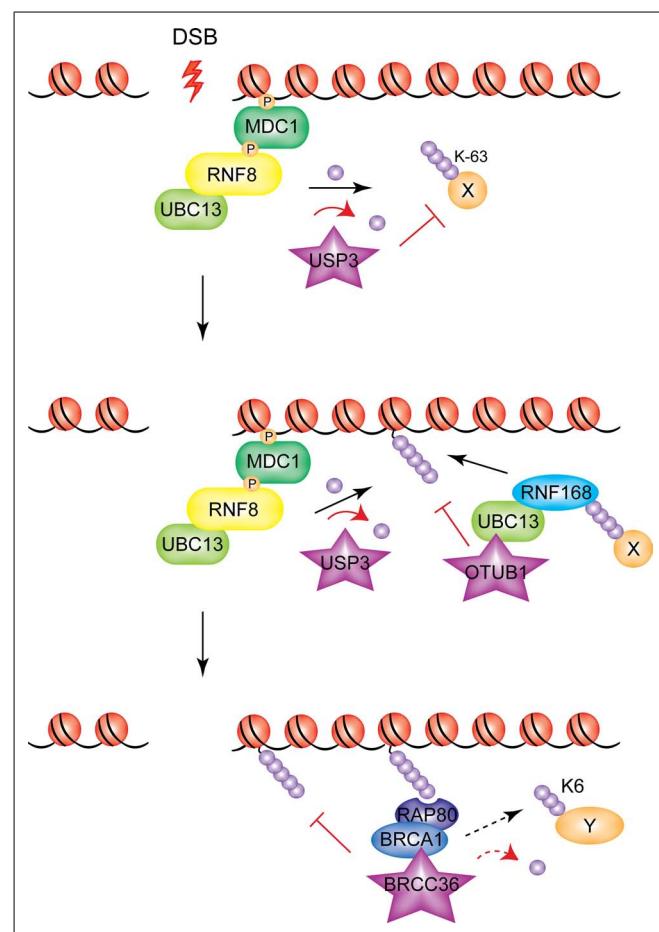
The human genome encodes nearly 100 DUBs that are divided into five classes based on their mechanism of catalysis (Nijman et al., 2005). The first four classes are papain-like cysteine proteases and include: the ubiquitin C-terminal hydrolases (UCHs), the ubiquitin-specific proteases (USPs), the ovarian tumor proteases (OTUs), and the Josephines. The fifth class is composed of the

JAB1/MPN/MOV34 metalloenzymes (JAMMs), which are zinc-dependent metalloproteases.

To date, four DUBs have been shown to counteract the action of RNF8/RNF168 on H2A/H2AX (see **Figure 3**): USP3, USP16 (ubiquitin-specific protease 3 and 16, respectively), BRCC36 (BRCA1/BRCA2-containing complex subunit 36) and OTUB1 (OUT domain ubiquitin aldehyde-binding 1).

USP3 has been shown to be a negative regulator of the RNF8/RNF168 pathway through its de-ubiquitylating activity (Nicassio et al., 2007). Overexpression of USP3, while having no effect on retention of RNF8 at DSBs, abolished the IR-induced focus formation of RNF168, RAP80, and 53BP1 (Doil et al., 2009), indicating that it de-ubiquitylates RNF8-targeted substrates.

USP16 is the major DUB for uH2A and acts as an antagonist of polycomb-dependent repression of gene expression (Joo et al., 2007). An ATM-dependent transcriptional silencing at DSBs has been reported, and seems to be at least partially mediated



by the ubiquitin mark deposited by RNF8/RNF168 at H2A. This transcriptional repression at DSBs can be alleviated by inhibition of ATM (ATMi). USP16 depletion could prevent ATM-mediated restoration of transcription, indicating that this DUB is responsible for de-ubiquitylation of uH2A at DSBs (Shanbhag et al., 2010). Whether the target for USP16-mediated de-ubiquitylation is uH2A deposited by RNF8/RNF168 or by other E3 ligases remains to be established (see “RING E3 Polycomb Proteins and the RNF8/RNF168 DSB Response Pathway” Section for more details).

The K63-specific BRCC36 is recruited to DSBs as part of the BRCA1-A complex (Shao et al., 2009). BRCC36-depletion increases DSB-associated ubiquitin and 53BP1 accumulation at DSBs. This increase is partially reversed following RNF8 depletion, consistent with a role for BRCC36 as negative regulator of the RNF8/RNF168 pathway via de-ubiquitylation of K63-linked ubiquitin chains. Although the reasons for the presence of a de-ubiquitylase function in the context of a complex that recognizes ubiquitin chains are not fully understood, it has recently been proposed that BRCC36 may function to remove K63-linked ubiquitin from one substrate, allowing BRCA1 to mediate the formation of K6-linked chains on the same or other substrates, thus providing a dynamic balance between formation and removal of ubiquitin signals at DSBs, potentially important for the subsequent steps of repair (Wu et al., 2012).

Finally, the DUB OTUB1 was also shown to be a negative regulator of the RNF8/RNF168 pathway, at the level of RNF168 (Nakada et al., 2010), albeit in a non-canonical fashion. Indeed, the ability of OTUB1 to inhibit RNF168-dependent ubiquitylation is independent of its catalytic activity, and is instead mediated by its binding to and inhibition of the E2 UBC13, which cooperates with RNF168, suggesting that E2 regulation could also represent a means to regulate the DDR pathway.

The concerted action of the DUBs described above has an important contribution in counteracting RNF8/RNF168-mediated ubiquitin signaling to prevent excessive spreading of damage signals at DSBs and to terminate the signal after repair processes have occurred (Figure 3). It is likely that additional DUBs acting on other RNF8/RNF168 targets and/or with different ubiquitin-conjugate specificity remain to be identified.

REGULATION OF PROTEIN STABILITY

An additional level of regulation that was recently uncovered acts at the level of RNF168 protein stability (Gudjonsson et al., 2012). Gudjonsson and colleagues show that RNF168 is a target for the HECT domain-containing E3s ligases, TRIP12 and UBR5, which reduce the pool of available nuclear RNF168 by targeting it for proteasomal-mediated degradation. Given the processive nature of RNF168 and its persistent self-recruitment to damaged DNA, this regulation is critical to limit the cellular levels of RNF168 and thus prevent excessive spreading of DNA damage-induced chromatin ubiquitylation and consequent hyper-accumulation of ubiquitin-mediated genome caretakers to undamaged regions of the genome (Figure 4). Indeed, in TRIP12/UBR5-depleted cells IR-induced 53BP1 foci are larger indicating hyper-accumulation and excessive spreading of these DDR factors along the chromatin. This has important consequences on DSB repair efficiency and consequent cell survival. Boosting chromatin ubiquitylation

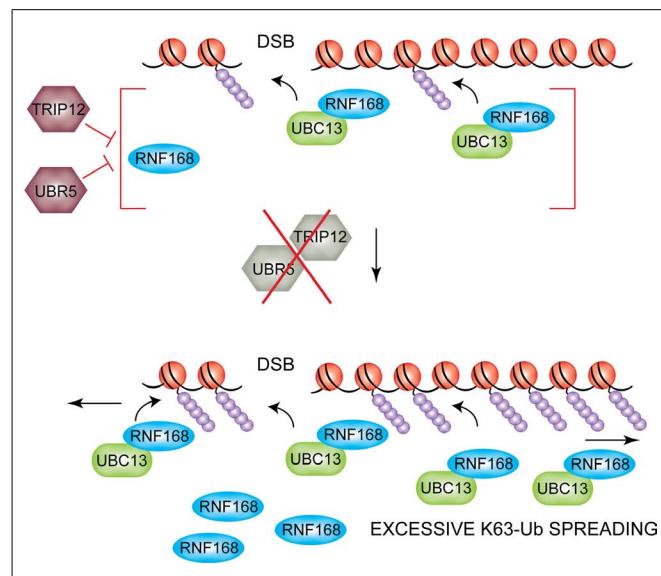


FIGURE 4 | TRIP12 and UBR5-mediated suppression of DSB-induced chromatin ubiquitylation. The HECT E3 ligases TRIP12 and UBR5 control the nuclear pool of RNF168 (top). If TRIP12 and UBR5 are depleted, hyper-accumulation of RNF168 triggers excessive spreading of ubiquitylated H2A/H2AX far away from the initial site of DNA lesion (bottom).

by inhibiting TRIP12/UBR5 enhances the repair efficiency of IR-induced DSBs by increasing rates of NHEJ, consistent with increased loading of 53BP1 on damaged chromatin. Interestingly, a more modest but nonetheless significant increase in HR rates could also be observed in the same experimental settings. The authors speculate that this could be due to hyper-accumulation of BRCA1 at sites of DNA lesions, which also is observed in TRIP12/UBR5-depleted cells and which could facilitate HR of unresolved DSBs close to the G2/M transition, when progressive chromatin condensation starts to displace NHEJ factors such as 53BP1. In addition to a faster and/or more efficient repair of DSBs, TRIP12/UBR5-depleted cells also displayed an improved short-term survival rate after irradiation, indicating that RNF168 overproducing cells could provide selective advantage to cancer cells that proliferate under increased genotoxic stress. Aberrations of both TRIP12 and UBR5 have been described in certain types of cancer (Clancy et al., 2003; O’Brien et al., 2008; Yoo et al., 2011), and elevated RNF168 protein levels and expanded 53BP1 foci were found in a subset of advanced human papillomavirus (HPV)-positive tumors (Gudjonsson et al., 2012), suggesting that the homeostasis of ubiquitin signaling achieved through regulation of RNF168 levels as illustrated here could be subverted during tumorigenesis.

Another HECT E3 ligase, HERC2, had been previously reported by the same group to have an opposite effect on RNF168 protein levels, by promoting RNF168 stability and therefore facilitating the assembly of repair factors to DSBs (Bekker-Jensen et al., 2010). In addition, the authors also showed that HERC2 was able to promote DSB signaling and repair by facilitating the assembly of RNF8 with its E2 partner UBC13, thus underscoring the importance of yet another E3 ligase, HERC2, in the RNF8/RNF168-dependent pathway in response to DSBs.

DOWNSSTREAM REGULATION BY RNF169 COMPETITIVE BINDING TO RNF8/RNF168 UBIQUITYLATED TARGETS

The RNF168 paralog RNF169 was recently identified as a novel RING E3 ligase with an unexpected negative role in the DNA damage-induced ubiquitin signaling response. RNF169 has a similar domain architecture to RNF168, including an N-terminal RING finger domain and UMI and MIU motifs and a C-terminal MIU motif (Panier et al., 2012; Poulsen et al., 2012). However, the nature of RNF169 recruitment to sites of damage is different to that observed for RNF168. Indeed, while RNF168 recruitment involves interaction with RNF8 targets through its N-terminal UBD region, the corresponding domain in RNF169 is dispensable for RNF169 localization. Instead, RNF169 accumulates at IRIFs in an RNF168-dependent manner by binding to RNF168-dependent ubiquitin products through its C-terminal MIU2. Functional studies demonstrated that while RNF169 is dispensable for ubiquitin-dependent protein assembly at DSB foci, its overexpression leads to impaired recruitment of 53BP1, BRCA1, RAP80, and RAD18 at these sites. Therefore, it was proposed that RNF169 competes with these DDR factors for binding to RNF168-dependent ubiquitin-modified chromatin (Panier et al., 2012; Poulsen et al., 2012). Indeed in a study on RNF169 the Mailand laboratory showed that overexpression of this E3 ligase stimulates HR efficiency while negatively impacting on the levels of NHEJ. By delaying and limiting the association of 53BP1 to DSBs while only having a mild effect on BRCA1 accumulation, the authors suggest that RNF169 might function to limit the use of NHEJ to repair the DNA lesions, thus channeling the repair toward the error-free HR pathway.

The data summarized above support a model in which the DNA damage-dependent ubiquitylation pathway is wired in a self-limiting circuit involving both positive and negative regulations, which allows histone ubiquitylation near the DNA lesions but at the same time prevents its excessive spreading to undamaged regions of the genome. Assembly and disassembly of this circuit allows for the timely accumulation of genome caretakers at sites of DNA damage to ensure a correct repair of the lesion.

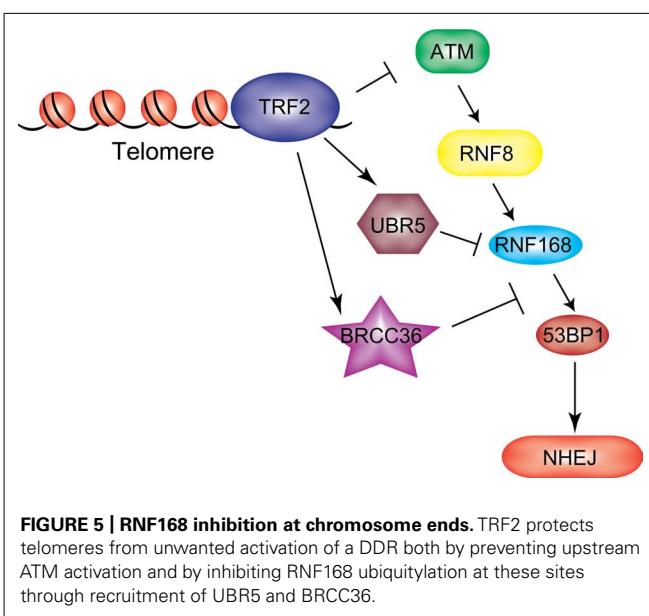
REGULATION OF RNF168 AT TELOMERES

Unprotected telomeres are perceived and processed by the cell as sites of DNA damage. This occurs upon attrition of telomeric DNA, or when specific components of shelterin are inhibited (Palm and de Lange, 2008). TRF2 (telomeric repeat binding factor 2) and POT1 (protection of telomeres 1) are involved in the suppression of the two major mammalian DDR pathways, mediated by ATM and ATR, respectively (Dench and de Lange, 2007). Deletion of TRF2 from mouse cells leads to ATM activation and ATM-dependent formation of DNA damage foci at telomeres. Telomere dysfunction-induced foci (TIFs) contain the same repertoire of repair factors detected at DSBs, such as γH2AX, MDC1, and 53BP1 (Takai et al., 2003). Dysfunctional telomeres are processed by the NHEJ pathway resulting in end-to-end fusions (Celli et al., 2006). Recent findings from our lab uncovered a two-step mechanism of end protection mediated by distinct portions of TRF2. The first step requires the TRFH domain, which is involved in preventing the initial step of the DDR response, while the second step is mediated by a short amino-acid motif in the Hinge domain

of TRF2, termed iDDR (inhibition of DDR) motif, that acts at the level of RNF168 (Okamoto et al., 2013). Expression of the iDDR region is sufficient to block the DDR signaling cascade at the level of RNF168 and prevent chromosome–chromosome fusions in TRF2-depleted cells. These data suggest that in mammalian cells repression of unwanted activation of the DDR at telomeres is achieved in part by inhibiting ubiquitin-dependent signaling at chromosome ends. The iDDR-dependent inhibition of RNF168 recruitment to dysfunctional telomeres requires the DUB enzyme BRCC36 and the ubiquitin ligase UBR5. This leads to a model in which RNF168 recruitment to telomeres is opposed by the concerted action of BRCC36 and UBR5 (Figure 5), underscoring again the importance of correctly regulating the RNF8/RNF168 pathway in order to ensure the maintenance of genomic stability.

CONTROL OF RNF8/RNF168 DURING VIRAL INFECTION

The DDR is involved in the cellular response to viral infection: many viruses induce signaling through the same cellular cascade activated by the DDR, while some parts of the DDR itself are manipulated by virally encoded proteins in order to prevent detrimental outcomes for viral infection (Weitzman et al., 2010, 2011). Indeed the DDR may have adverse reactions on viral infection through checkpoint activation, recruitment of repressive factors and processing of viral DNA. Many viruses interact with the ubiquitin–proteasome system to block immune response and promote virus replication (Blanchette and Branton, 2009; Isaacson and Ploegh, 2009; Randow and Lehner, 2009; Viswanathan et al., 2010). E3 enzymes confer the majority of the substrate specificity to the ubiquitylation cascade; therefore, it is not surprising that this step is often targeted by viral proteins. One such example is the viral E3 ligase ICP0, encoded by the herpes simplex virus type 1 (HSV-1). ICP0 is a RING finger E3 that induces proteasomal degradation of several cellular proteins including the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs), components of promyelocytic leukemia (PML) nuclear bodies, and



centromeric proteins (Everett et al., 1998, 1999; Parkinson et al., 1999; Lomonte et al., 2001; Gu and Roizman, 2003; Lomonte and Morency, 2007). Work from the Weitzman laboratory has uncovered that ICP0 also targets RNF8 and RNF168, consequently blocking the DDR pathway at this level to prevent downstream repair proteins from accumulating at sites of cellular damage (Liley et al., 2010). ICP0 expression promotes degradation of both RNF8 and RNF168 independently in a RING-dependent manner. RNF8 was shown to be targeted by ICP0 through the exploitation of a cellular phosphorylation-based strategy in a more recent study from the same laboratory (Chaurushya et al., 2012). ICP0 is phosphorylated by the cellular kinase CK1 on T67, mimicking the phospho-sites induced on MDC1 during the DDR signaling. This phospho-site directly binds the RNF8 FHA domain, competing with the interaction with MDC1, and is responsible for RNF8 degradation.

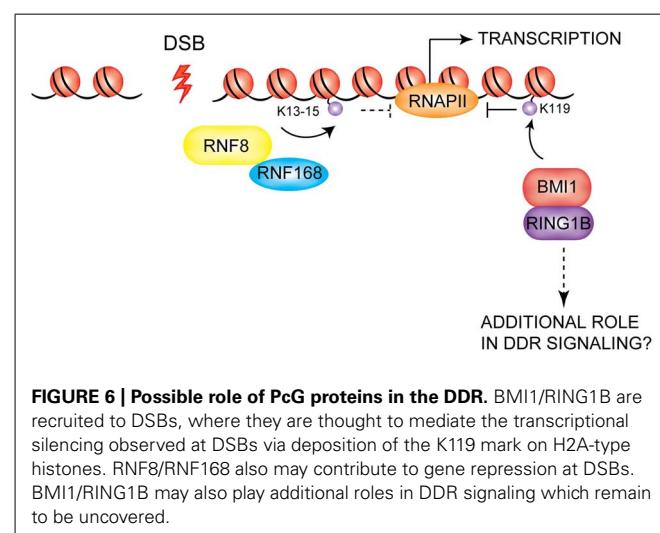
Given that viruses have evolved to target key convergence points in cellular pathways, the data described above once again highlight the importance of the RNF8/RNF168 pathway as a nodal point in the DDR pathway that needs to be tightly regulated.

RING E3 POLYCOMB PROTEINS AND THE RNF8/RNF168 DSB RESPONSE PATHWAY

Recent studies have uncovered a function for PcG proteins in the cellular response to DNA damage that may partially overlap with that of RNF8/RNF168. PcG proteins are chromatin-associated proteins that control a variety of cellular processes such as maintenance of cellular identity, proper embryonic development, and cell differentiation and proliferation (Pietersen and van Lohuizen, 2008; Bracken and Helin, 2009; Sauvageau and Sauvageau, 2010; Surface et al., 2010). Given the well-established role of PcG proteins in gene silencing, it is likely that PcGs contribute to the transcriptional repression observed at DSBs. Gene silencing at sites of DNA lesions was shown to be ATM-dependent and able to spread across kilobases of DNA *in cis* to the damage (Cuozzo et al., 2007; O'Hagan et al., 2008; Shanbhag et al., 2010). An important effector of this phenomenon is H2A ubiquitylation: the levels of uH2A at DSBs are strongly reduced upon ATM inhibition after DNA damage induction (while no effect is seen on K63-linked ubiquitin species at the same site) and this reduction correlates with a reversal of repression of transcription (Shanbhag et al., 2010). It has been demonstrated that expression of a mutant form of H2A that cannot be monoubiquitylated at K119 partially rescues transcription. A heterodimer of the E3 ligases BMI1 and RING1B of PRC1 catalyzes histone H2A monoubiquitylation at K119 (Wang et al., 2004; Cao et al., 2005), promoting gene silencing partly exerted by controlling the RNA polymerase II-mediated elongation phase of transcription (Stock et al., 2007). Thus, BMI1/RING1B may be the E3s responsible for the deposition of the H2AK119Ub repressive mark at sites of DNA lesions. Residues K13–15 of H2A/H2AX were recently identified as the target lysines for RNF8/RNF168 (Mattioli et al., 2012), thus distinguishing the RNF8/RNF168 target from that of BMI1/RING1B (K119), suggesting that ubiquitylation of these two sites provides independent signals in the response to DSBs. In their study, Shanbhag and colleagues found that co-depletion of RNF8 and RNF168

also lead to a partial rescue of transcription levels at DSBs in cells treated with an ATM inhibitor after DNA damage induction. Whether RNF8/RNF168-dependent deposition of uH2A on K13–15 also directly plays a role in the induction of gene silencing at DSBs remains to be established (Figure 6). In this context, it will be important to elucidate the functional cross-talk between the RNF8/RNF168 ubiquitin pathway and the PcG proteins to determine if BMI1/RING1B also play a role in coordinating DSB signaling in addition to their hypothesized role in gene silencing at these sites. Several laboratories have recently shown that BMI1 and RING1B accumulate at sites of DSBs generated by IR or laser micro-irradiation, although there are some discrepancies between the different reports in terms of the effect of depletion of BMI1 on the recruitment of downstream DDR effectors and consequently on DNA repair (Chou et al., 2010; Facchino et al., 2010; Ismail et al., 2010; Chagraoui et al., 2011; Ginjala et al., 2011). Further studies will be required to elucidate the precise role for the PcG proteins in the DDR pathway.

It will also be interesting to determine whether DUBs have a role in regulating the activity of PcG E3 ligases in the context of the DSB response pathway, analogous to their contribution to the regulation RNF8/RNF168 as described above. The DUBs USP3 and USP11 are components of the PRC1 complex and are known to regulate BMI1 and MEL18 (another component of PRC1) turnover and abundance (Maertens et al., 2010). A novel PcG complex, polycomb repressive de-ubiquitinase (PR-DUB) has recently been described in *Drosophila* and is conserved in humans. It contains the BRCA1-complex associated protein 1 (BAP1), a tumor suppressor protein with DUB activity that is involved in de-ubiquitylation of H2AK119Ub (Ventii et al., 2008; Scheuermann et al., 2010). In addition, depletion of USP16, which acts as an antagonist of polycomb-dependent repression of gene expression (Joo et al., 2007), was shown to prevent the reversal of transcriptional silencing and decrease of uH2A levels observed at DSBs upon ATM inhibition or cessation of DNA damage. This indicates that USP16 is responsible for de-ubiquitylation of uH2A at DSBs (Shanbhag et al., 2010). Whether USP16 targets the products of



RNF8/RNF168- and/or BMI1/RING1B-dependent ubiquitylation remains to be established.

THE RNF8/RNF168 PATHWAY AND SUMO-DEPENDENT SIGNALING

Increasing evidence supports an important role for sumoylation in promoting the response to DSBs (reviewed in Bekker-Jensen and Mailand, 2011; Praefcke et al., 2012). Similarly to ubiquitylation, sumoylation is a process through which a SUMO moiety is covalently attached to a substrate protein. SUMO1, SUMO2/3, and the SUMO E3 ligases PIAS1 and PIAS4 accumulate at DSBs; PIAS1 and PIAS4 were shown to be responsible for sumoylation of 53BP1 as well as BRCA1 and their consequent accrual and/or retention at IRIFs (Galanty et al., 2009; Morris et al., 2009). Furthermore, a recent study from the Mailand group demonstrated that sumoylation of HERC2 and RNF168 by PIAS4 promotes ubiquitin-mediated protein assembly at DSB-surrounding chromatin, indicating that sumoylation is involved in regulation of the RNF8/RNF168 pathway (Danielsen et al., 2012). RNF8 or RNF168 depletion does not prevent PIAS1/4 accumulation at DSBs, but it does reduce the accrual of SUMO1 and SUMO2/3 to the damage sites. Thus the mechanism through which PIAS1 and PIAS4 are recruited to DSBs is independent of RNF8/RNF168, while presence of sumoylated protein species at damaged chromatin is downstream of RNF8/RNF168, likely because these ligases are in first instance required for recruitment of target proteins such as BRCA1 and 53BP1, which are subsequently sumoylated.

An additional link between the RNF8/RNF168 pathway and sumoylation is provided by the findings that the SUMO-targeted ubiquitin ligase (STUBL) RNF4, which is also recruited to DSBs, generates hybrid SUMO-ubiquitin chains at sites of DNA lesions which are critical for the recruitment of RAP80 and BRCA1 to the damaged chromatin and for consequent DNA repair (Prudden et al., 2007; Galanty et al., 2012; Guzzo et al., 2012). RNF4 is not required for RNF8/RNF168 recruitment to DSBs, but its depletion leads to delayed clearance of RNF8, RNF168, and 53BP1 from DSB foci and, strikingly, to a significant decrease in K63-linked ubiquitin chains that are deposited at DSBs (Yin et al., 2012). One possibility is that the mixed SUMO-ubiquitin chains generated by RNF4 are required to amplify the ubiquitin signal deposited by RNF8/RNF168. Further studies are required to understand the exact role of RNF4 at DSBs and how the complex interplay between RNF8/RNF168-dependent ubiquitin signaling and

SUMO signaling at sites of DNA lesions contributes to DNA repair and cell survival.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The DDR and repair pathways are of vital importance to correct damages in the genome and ensure genomic stability. A wide variety of human genome instability syndromes exist whose underlying cause is the inactivation of DDR and repair genes (reviewed in Kerzendorfer and O'Driscoll, 2009). A paradigm for such syndromes is ataxia-telangiectasia (A-T), caused by a biallelic mutation in ATM (Savitsky et al., 1995). A-T symptoms are progressive neurodegeneration, immune dysfunction, hypersensitivity to IR, and marked cancer predisposition (Lavin and Shiloh, 1997). Inaccurate repair of DSBs can lead to chromosomal rearrangements that promote tumorigenesis (Jeggo and Lobrich, 2007). The present review focused on the multiplicity of regulation mechanisms that have evolved to tightly control one important node of the DDR signaling cascade, the RNF8/RNF168 pathway. Mutations of RNF168 are associated with the RIDDLE syndrome, which is characterized by defects in repairing DSBs (Stewart et al., 2009). It will be important to determine if RNF8/RNF168 activity opposes tumor formation and whether the DDR ubiquitylation pathway could represent a novel therapeutic target for cancer treatment. The multiple regulations of the RNF8/RNF168 pathway represent potential therapeutic targets, for example, in situations in which compromised ubiquitylation activity has a radioprotective function in cancer cells. In these cases the inhibition of specific DUBs may represent a viable therapeutic strategy.

Polycomb group-mediated repression of tumor suppressor genes is causally linked to cancer development (Bracken and Helin, 2009). The recent finding that PcG proteins may be involved in the response to DSBs provides an incentive to develop small-molecule inhibitors of PcG in order to treat cancer in combination with standard treatment strategies such as chemotherapy or radiotherapy, which induce DNA damage.

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Reading, writing, and repair: the role of ubiquitin and the ubiquitin-like proteins in DNA damage signaling and repair

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Genomic instability is both a hallmark of cancer and a major contributing factor to tumor development. Central to the maintenance of genome stability is the repair of DNA damage, and the most toxic form of DNA damage is the DNA double-strand break. As a consequence the eukaryotic cell harbors an impressive array of protein machinery to detect and repair DNA breaks through the initiation of a multi-branched, highly coordinated signaling cascade. This signaling cascade, known as the DNA damage response (DDR), functions to integrate DNA repair with a host of cellular processes including cell cycle checkpoint activation, transcriptional regulation, and programmed cell death. In eukaryotes, DNA is packaged in chromatin, which provides a mechanism to regulate DNA transactions including DNA repair through an equally impressive array of post-translational modifications to proteins within chromatin, and the DDR machinery itself. Histones, as the major protein component of chromatin, are subject to a host of post-translational modifications including phosphorylation, methylation, and acetylation. More recently, modification of both the histones and DDR machinery by ubiquitin and other ubiquitin-like proteins, such as the small ubiquitin-like modifiers, has been shown to play a central role in coordinating the DDR. In this review, we explore how ubiquitination and sumoylation contribute to the “writing” of key post-translational modifications within chromatin that are in turn “read” by the DDR machinery and chromatin-remodeling factors, which act together to facilitate the efficient detection and repair of DNA damage.

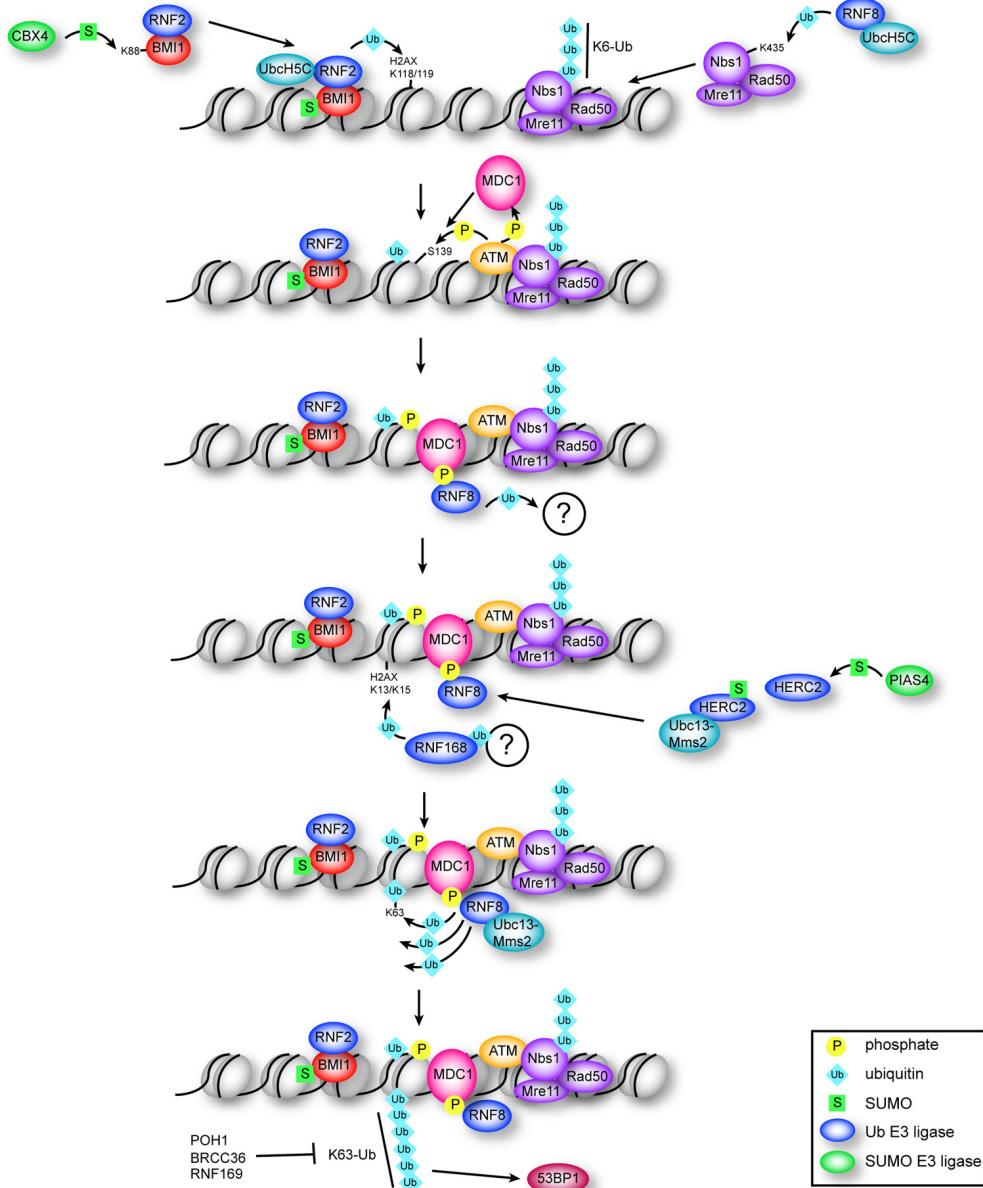
Keywords: ubiquitin, SUMO, DNA repair, E3 ligase, RNF8, MDC1, H2AX

INTRODUCTION

Genomic stability is continuously being threatened by insults arising from both endogenous (metabolic) and exogenous (environmental) sources (Panier and Durocher, 2009; van Attikum and Gasser, 2009). The result can be a variety of DNA lesions including damaged or modified bases, intra-strand cross-links, as well as single- and double-strand DNA breaks (Ciccia and Elledge, 2010). DNA double-strand breaks (DSBs) represent one of the most cytotoxic DNA lesions (Wyman and Kanaar, 2006). DSBs can be produced during normal cellular metabolism and DNA replication, as well as exogenously through exposure to ionizing radiation (IR) or chemical mutagens (Ciccia and Elledge, 2010). DNA DSBs are repaired either by non-homologous end-joining (NHEJ), which occurs at any time in the cell cycle, or by homologous recombination (HR), which occurs predominately in S and G2 phase, peaking in mid-S phase (Karanam et al., 2012). If not properly repaired, DNA DSBs can lead to a spectrum of mutations that can trigger cell death if normal checkpoint function is intact, or induce cellular transformation by activating oncogenes or disrupting tumor suppressor function (Wyman and Kanaar, 2006).

As a consequence, to maintain genomic stability a multi-branched, highly coordinated signaling cascade is initiated following the induction of even a single DNA DSB (Huang et al., 1996). This signaling cascade, termed the DNA damage response (DDR)

integrates several cellular responses including DNA repair, cell cycle checkpoint activation, transcriptional regulation, or apoptosis if damage proves too severe (Bao, 2011). One of the hallmarks of the cellular response to DNA DSBs is the focal accumulation of many of the DDR proteins at the break site (van Attikum and Gasser, 2009). This assembly of repair factors on DNA DSBs occurs in a highly regulated manner according to a strict hierarchy and is reliant on the phosphorylation of the key histone variant H2AX (termed γ -H2AX; Figure 1; Rogakou et al., 1998; Paull et al., 2000). Following DNA DSB induction, H2AX is rapidly phosphorylated by a set of phosphoinositide-3-kinase-related kinases: ATM (ataxia telangiectasia mutated), ATR (ATM- and RAD3-related), and DNA-PK (DNA-dependent protein kinase; Ward and Chen, 2001; Stiff et al., 2004) and is crucial for rapid amplification of the DNA damage signal. MDC1 (mediator of DNA damage checkpoint 1), a key mediator of the DDR, binds directly to γ -H2AX and recruits the MRE11/RAD50/NBS1 (MRN) complex to break sites (Lukas et al., 2004; Stucki et al., 2005). The MRN complex in turn can further stimulate ATM activity leading to rapid spreading of γ -H2AX around the DNA break, and therefore the amplification of the DDR signal (Uziel et al., 2003; Lee and Paull, 2005). In addition, γ -H2AX is crucial for the effective recruitment and retention of many DNA repair enzymes at DNA DSBs, including 53BP1, BRCA1, and RAD51 (Paull et al., 2000; Nakamura et al., 2010) as well as chromatin-remodeling complexes such as SWI1

**FIGURE 1 | Multiple roles for ubiquitin and SUMO in the early DDR.**

Constitutive ubiquitination of NBS1 by RNF8–UbcH5c is required for localization of the MRN complex to DNA breaks. BMI1–RNF2 is targeted to DSBs by damage-induced CBX4-mediated sumoylation of BMI1. RNF2–BMI1–UbcH5c monoubiquitinates H2AX on K118/K119, which is required for recruitment of ATM and efficient production of γ-H2AX. MDC1 binds γ-H2AX and is phosphorylated by ATM, which recruits RNF8. RNF8

ligase activity is required (through an unknown mechanism) to recruit RNF168, which ubiquitinates H2AX at K13/K15. RNF8 catalyzes K63 chains on K13/K15-ubiquitinated H2AX through association with Ubc13–Mms2, which depends on interaction of RNF8 with sumoylated HERC2. Formation of K63 chains promotes recruitment of 53BP1 through an unknown mechanism, and is antagonized by RNF169 and by the DUBs POH1 and BRCC36.

and INO80 (Downs et al., 2004; Morrison et al., 2004; van Attikum et al., 2004), resulting in the accumulation of a high concentration of repair factors in the vicinity of a break. The recruitment of these factors to the site of DNA DSBs is complicated by the fact that the physiological substrate upon which repair must occur is not naked DNA, but rather DNA complexed with histone proteins in the form of chromatin. Furthermore, the compaction of eukaryotic chromatin is variable, with DNA being packaged as

either euchromatin or heterochromatin. Euchromatin represents loosely packed, transcriptionally active gene-rich regions, while heterochromatin is generally characterized by highly repetitive regions that are tightly compacted and are transcriptionally silent (Gelato and Fischle, 2008). The differential compaction of DNA into either euchromatin or heterochromatin thus serves to control access of various proteins to the underlying DNA, regulating key cellular processes such as transcription, DNA replication,

and repair (Groth et al., 2007; Li et al., 2007). Accordingly, the interplay between chromatin and DNA repair factors plays a central role in the cellular response to DSBs, and modulation of chromatin structure is critical for mediating access of repair proteins to underlying DNA lesions (Costelloe et al., 2006). To overcome the physical barrier posed by chromatin structure, a variety of histone modifying enzymes and chromatin-remodeling complexes are recruited to break sites following DNA damage to facilitate binding of DNA repair proteins (Dinant et al., 2008). Histones are also subject to a vast array of post-translational modifications including phosphorylation, methylation, acetylation, ubiquitination, and sumoylation. Together, these modifications can influence the structure of chromatin directly, for example by impacting the stability of individual nucleosomes, or indirectly by creating or eliminating binding sites for non-histone proteins, such as ATP-dependent chromatin remodelers that can in turn facilitate changes in chromatin organization (Saha et al., 2006).

Whereas the influence of acetylation, methylation, and phosphorylation on chromatin structure and the impact of these modifications on DNA repair has been extensively investigated, we are only now beginning to appreciate that a much larger spectrum of protein modifications is at play during the DDR. In particular, the modification of both chromatin and DNA repair factors by ubiquitin and the small ubiquitin-like modifiers (SUMOs) has recently been shown to play a central role in the detection and repair of DNA DSBs. Here we will explore how ubiquitination and sumoylation control key post-translational modifications within chromatin that are recognized by DNA repair and chromatin-remodeling factors, which act together to facilitate the efficient detection and repair of DNA damage (summarized in **Figure 1**).

THE UBIQUITIN-LIKE FAMILY OF PROTEINS: MODULATING ASSEMBLY OF PROTEIN COMPLEXES THROUGH COVALENT AND NON-COVALENT INTERACTIONS

Ubiquitin and SUMO are two members of a family of ubiquitin-like proteins (UBLs) that are conjugated to target proteins post-translationally (Hochstrasser, 2009). Ubiquitin and SUMO can be attached to lysine residues in target proteins through an isopeptide bond, and also bind non-covalently to interacting partners at specific domains called ubiquitin-binding domains (UBDs) and SUMO-interaction motifs (SIMs), respectively.

The conjugation systems for ubiquitin and SUMO are mediated by a set of enzymes specific for each UBL (Johnson and Blobel, 1997). The mechanism of ubiquitin conjugation is summarized below. Carboxy-terminal residues in the primary translation product of ubiquitin are removed by specific proteases to expose a diglycine motif that is ultimately linked to a nucleophilic side chain (usually lysine) in the target protein. Catalysis occurs in a sequential manner by three distinct classes of enzymes: an activating enzyme (E1), conjugating enzyme (E2), and ligase (E3). Ubiquitin is first activated in an ATP-driven reaction that forms a high-energy thioester bond between its terminal carboxylate and a cysteine residue in the E1. Ubiquitin is transferred via transthioesterification to the active site cysteine residue of the E2, and then is generally conjugated to a lysine residue in the target protein

with the assistance of an E3 ligase. Ubiquitin E3 ligases are divided into two families, the largest of which is the really interesting new gene (RING) E3 family, for which there are more than 600 potential members in mammals (Li et al., 2008). RING domain ligases bridge the interaction between E2-ubiquitin conjugates and the target protein, providing an orientation favorable to catalysis. The smaller family of ubiquitin E3s (~30 in mammals; Metzger et al., 2012) are the HECT (homologous to the E6AP carboxyl terminus) ligases, through which an additional thioester intermediate is formed during transfer of ubiquitin to the substrate (Rotin and Kumar, 2009).

Sumoylation occurs by a similar mechanism as ubiquitination, with some notable distinctions. Mammals encode ~40 ubiquitin E2s, but only one SUMO E2, Ubc9 (Kerscher et al., 2006; Gareau and Lima, 2010). Several types of SUMO E3s have been characterized to date, one family containing an SP-RING domain that is analogous to the RING domain of ubiquitin E3s. Covalent attachment of ubiquitin and SUMO to target proteins is reversible, and removal is catalyzed by de-ubiquitinating enzymes (DUBs) and SUMO-specific proteases (sentrin-specific proteases, SENPs), respectively. Although vertebrates encode just a single ubiquitin protein, there are at least three major isoforms of SUMO that are relevant for DNA repair in mammals, encoded by separate genes, SUMO-1, SUMO-2, and SUMO-3 (Citro and Chiocca, 2013). There is also evidence of a fourth SUMO paralog in humans called SUMO-4; however, it appears to function in the cytoplasm and its expression is limited to kidney, spleen, and lymph tissue (Bohren et al., 2004; Guo et al., 2004). Due to the nearly indistinguishable function and close similarities in sequence between SUMO-2 and -3 (~97% identical), they are commonly referred to as SUMO-2/3 in the literature (Bayer et al., 1998). Ubiquitin is a target of itself, and can form branched chains at any of its seven lysine residues (K6, K11, K27, K29, K33, K48, K63) and linear chains through its amino-terminal methionine amino group (Husnjak and Dikic, 2012; Walczak et al., 2012). The most well-known function of ubiquitin is to target proteins for proteasomal degradation, which is signaled by K48-linked chains. SUMO-1 is mostly associated with mono-sumoylation whereas SUMO-2 and -3, like ubiquitin, can form poly-SUMO chains via K11, with SUMO-2 forming chains more readily than SUMO-3, and SUMO-1 potentially acting as a SUMO chain terminator (Tatham et al., 2001).

Although ubiquitination is typically associated with proteasomal degradation, both ubiquitin and SUMO conjugation can serve to modulate the interacting partners of the modified protein, in many cases by enabling recognition by proteins containing UBDs and SIMs, respectively. UBDs have many different subtypes, with those relevant in DDR-pathway proteins including MIU (motif interacting with ubiquitin), UIM (ubiquitin interacting motif), and UMI (UIM and MIU-related UBD; Hicke et al., 2005; Dikic et al., 2009; Pinato et al., 2011; Husnjak and Dikic, 2012). Most SIMs are characterized by a hydrophobic core often flanked by acidic residues (Song et al., 2004; Hannich et al., 2005; Hecker et al., 2006). Specificity of UBD-containing proteins can be conferred by tandem UBDs that recognize a specific ubiquitin chain topology, and also by additional peptide motifs to which the UBDs are juxtaposed (Husnjak and Dikic, 2012; Panier et al., 2012).

Ubiquitin and SUMO have critical functions in DNA repair, and protein conjugates of ubiquitin and SUMO are observed at sites of DNA DSBs (Galanty et al., 2009; Morris et al., 2009; Stewart et al., 2009). Both K48- and K63-linked ubiquitin chains are detected at DSBs immediately after damage, although K63-linked chains persist for a much longer time (Feng and Chen, 2012). Ubiquitin conjugates are observed as soon as 15 s following DNA damage. This initial wave is mediated by the ubiquitin E3 ligase cased hole formation resistivity (CHFR), that binds to poly(ADP-ribosyl)ated proteins, which rapidly accumulate at DNA breaks (Liu et al., 2012). A second wave of ubiquitination occurs about one minute after damage, and is mediated by the E3 ring finger protein 8 (RNF8; discussed below; Liu et al., 2012). SUMO-1 and SUMO-2/3 are also observed at breaks immediately after damage, though SUMO-1 accrual may lag slightly behind SUMO-2/3 (Galanty et al., 2009; Hu et al., 2012). SUMO persists at breaks for several hours after damage (Galanty et al., 2009). Ubiquitin and SUMO serve to recruit and assemble repair factors at sites of DNA damage through interaction with UBDs and SIMs,

respectively. Recent advances in the function of ubiquitin and SUMO during the repair of DNA DSBs will be discussed in more detail below, and key substrates of sumoylation and ubiquitination involved in DNA DSB repair are summarized in **Tables 1** and **2**, respectively.

MONOUBIQUITINATION OF H2AX BY RNF2: AN EARLY STEP IN DNA REPAIR

One of the earliest events in DSB repair is the recruitment of ATM kinase to the site of the break, where it phosphorylates numerous targets, in particular histone H2AX at S139 to form γ-H2AX. Recent reports illustrate a role for the E3 ubiquitin ligase RNF2 (RING1b/RING2) in ATM recruitment. RNF2 and its adaptor protein BMI1 (B lymphoma Mo-MLV insertion region 1 homolog) are RING domain-containing proteins of the Polycomb repressive complex 1 (PRC1) (Sparmann and van Lohuizen, 2006) and catalyze monoubiquitination of histone H2A (Wang et al., 2004; Cao et al., 2005; Buchwald et al., 2006). Approximately 5–15% of H2A is constitutively monoubiquitinated (Levinger

Table 1 | Sumoylation targets in the early DDR.

SUMO target	Isoform	Site(s)	E3(s)	Proposed function	Reference
BMI1	SUMO-1	K88	CBX4	Accumulation of BMI1 at DSBs	Ismail et al. (2012)
HERC2	SUMO-1	nd	PIAS4	Promotes binding to RNF8	Danielsen et al. (2012)
RNF168	SUMO-1	nd	PIAS4	Maintain RNF168 levels	Danielsen et al. (2012)
53BP1	SUMO-1	nd	PIAS4	Unknown	Galanty et al. (2009)
BRCA1	SUMO-1, SUMO-2/3	nd	PIAS1, PIAS4	Stimulates ligase activity	Galanty et al. (2009); Morris et al. (2009)
MDC1	SUMO-1, SUMO-2/3	K1840	PIAS4	Signal for RNF4-mediated ubiquitination	Luo et al. (2012)
RAP80	SUMO-1, SUMO-3	nd	nd	unknown	Yan et al. (2007)
RPA70	SUMO-2/3	K449, K577	nd	Facilitates RAD51 recruitment	Dou et al. (2010)

nd, not determined.

Table 2 | Ubiquitination targets in the early DDR.

Ubiquitin target	Type of linkage	Site(s)	E2	E3	Proposed function	Reference
PARP1	K48-Ub chains, K63-Ub chains	K88	UbcH5C Ubc13	CHFR DSB sites	Displacement of PARP1 from DSB sites	Liu et al. (2012)
H2AX	Mono-Ub	K119, K120	UbcH5C	RNF2–BMI1	Required for recruitment of ATM	Facchino et al. (2010); Ismail et al. (2010), Bentley et al. (2011); Ginjala et al. (2011), Wu et al. (2011)
H2AX	Mono-Ub, some K63-Ub chains	K13, K15	UbcH5C	RNF168	Priming for RNF8-mediated ubiquitination	Gatti et al. (2012); Mattioli et al. (2012)
Ub-H2AX (K13/15)	K63-Ub chains	K13, K15	Ubc13	RNF8	Important for 53BP1 recruitment	Mattioli et al. (2012)
MDC1	K63-Ub chains	K1977	Ubc13	nd	Recruits RAP80	Strauss et al. (2011)
SUMO-MDC1 (K1840)	K48-Ub chains	nd	nd	RNF4	Degradation of MDC1	Luo et al. (2012)
NBS1	K6-Ub chains	K435	UbcH5C	RNF8	Recruits NBS1 to DSBs	Lu et al. (2012)
JMJD2A	K48-Ub chains	nd	UbcH5C	RNF8/RNF168	Proteasomal degradation, to expose H4K20me2	Mallette et al. (2012)

nd, not determined.

and Varshavsky, 1980; West and Bonner, 1980) and serves to repress transcription through inhibition of RNA polymerase II transcription elongation (Zhou et al., 2008). RNF2–BMI1 was also shown to play a role in DNA repair, based on observations that depletion of either RNF2 or BMI1 causes increased sensitivity to IR, and a delayed DDR (Facchino et al., 2010; Ismail et al., 2010; Ginjala et al., 2011; Pan et al., 2011; Wu et al., 2011). Following DNA damage, RNF2–BMI1 catalyzes monoubiquitination of H2AX at K119 and K120 (K118 and K119 in H2A; Bergink et al., 2006; Ginjala et al., 2011; Pan et al., 2011; Wu et al., 2011). This modification is required for recruitment of ATM to sites of damage, and consequently, is necessary for efficient formation of γ -H2AX (Pan et al., 2011; Wu et al., 2011). Since the kinase DNA-PK is functionally redundant to ATM in phosphorylation of H2AX (Stiff et al., 2004), knock-down of RNF2 in the presence of a DNA-PK inhibitor is required to completely ablate formation of γ -H2AX (Pan et al., 2011).

BMI1 tethers RNF2 to DNA, and associates more stably with damaged compared to undamaged chromatin (Ismail et al., 2010). Computational models based on a recently derived crystal structure of BMI1–RNF2–ubiquitin-conjugating enzyme H5c (UbcH5c) suggest that the complex binds to both nucleosomal DNA and histone H4 (Bentley et al., 2011), while initial recruitment of RNF2–BMI1 to DSBs is dependent on sumoylation of BMI1 (Ismail et al., 2012). The PRC1 complex member CBX4 (chromobox homolog 4) promotes sumoylation (SUMO-1) of BMI1 at K88, with the BMI1 K88R mutant failing to be recruited to repair foci (Ismail et al., 2012). Although BMI1 is required for initial recruitment of ATM, ATM is required for sustained localization of BMI1 at breaks, which is important for efficient HR (Ginjala et al., 2011). Further experimentation will be required to elucidate the mechanism by which sumoylation mediates RNF2–BMI1 assembly at DSBs, and how H2AX ubiquitination enables recruitment of ATM. Initial studies indicate that ubiquitination of H2A may weaken interaction with DNA, destabilizing the nucleosome (Li et al., 1993). Consistent with this hypothesis, K118 and K119 in H2A form hydrogen bonds with DNA that would be disrupted by conjugation to ubiquitin (Biswas et al., 2011). However, nucleosome stability has yet to be directly implicated in recruitment of ATM.

MULTIPLE CATALYTIC ROLES FOR THE UBIQUITIN E3 LIGASE RNF8 IN DNA REPAIR

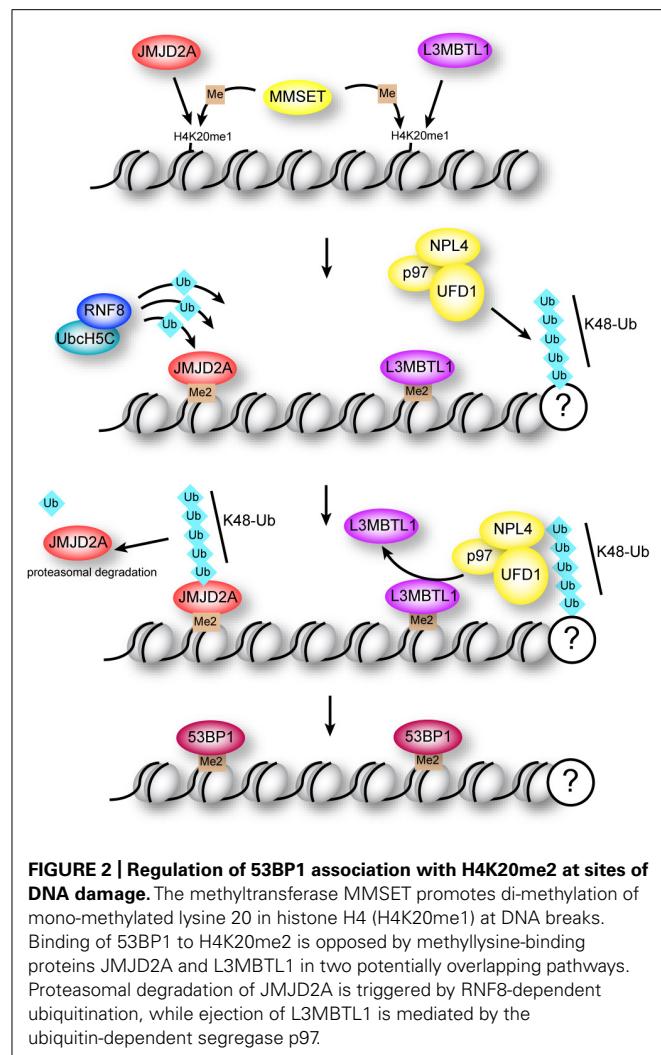
While H2A monoubiquitination is an important early step in the DDR, extensive ubiquitin chains linked at K48 and K63 are also observed in the vicinity of DNA breaks (Meerang et al., 2011). K63 chains are particularly important in recruitment of downstream DDR repair proteins, such as RAP80 and 53BP1 (Ciccia and Elledge, 2010; Polo and Jackson, 2011; Hu et al., 2012). The major E3 ligase responsible for catalyzing formation of these chains is RNF8. Following formation of γ -H2AX, ATM phosphorylates MDC1, creating a binding site for the forkhead-associated (FHA) domain of RNF8 (Huen et al., 2007; Mailand et al., 2007; Marteijn et al., 2009). RNF8 is required for recruitment of another E3 ubiquitin ligase, RNF168, to repair foci (Doil et al., 2009; Stewart et al., 2009). RNF8 and RNF168 act in concert to catalyze non-proteolytic K63-linked ubiquitin

chains conjugated to H2AX on residues K13 and K15 (Gatti et al., 2012; Mattioli et al., 2012). These residues are located on the opposite side of the nucleosome as the sites targeted for monoubiquitination by RNF2. Polyubiquitination of H2AX is required for proper DNA DSB signaling, as expression of ligase-dead RNF168 affects recruitment of downstream DDR repair factors, including RAP80, BRCA1, and 53BP1 (Mattioli et al., 2012).

Despite the preliminary *in vitro* biochemical evidence pointing to RNF8 mediating the initial “priming” ubiquitination of H2AX followed by RNF168 during the DDR, new evidence has come to light that challenges this hierarchy in the establishment of the K63 ubiquitin chains on H2AX. Although RNF8 can ubiquitinate free H2A *in vitro*, and despite the fact that RNF168 recruitment to DNA breaks requires both the catalytic activity of RNF8, as well as the MIU domains of RNF168 (Doil et al., 2009; Stewart et al., 2009), nucleosomal H2A is a substrate of RNF168 and cannot be modified by RNF8 (Gatti et al., 2012; Mattioli et al., 2012). Thus, RNF8 efficiently adds K63-linked ubiquitin chains to H2A following initial ubiquitination by RNF168 (Mattioli et al., 2012). Therefore, the requirement of RNF8 catalytic activity for RNF168 recruitment may reflect the contribution of the RNF8-mediated ubiquitination of a non-nucleosomal protein (Mattioli et al., 2012). While this protein has not yet been identified, RNF8 has been shown to target other DDR-pathway proteins for ubiquitination, including the MRN component NBS1 (Lu et al., 2012), and JMJD2A, which obstructs binding of 53BP1 to dimethylated K20 in histone H4 (H4K20me2; Mallette et al., 2012; **Figure 2**, and discussed below). Ubiquitination of NBS1 is required for recruitment of both NBS1 and MRE11 to DNA DSBs, and deficient NBS1 ubiquitination impairs the HR repair pathway (Lu et al., 2012).

Supporting the hypothesis that RNF8 is responsible for H2AX polyubiquitination, RNF8 interacts with the E2 Ubc13–Mms2, the only E2 capable of forming K63-linked ubiquitin chains (Hofmann and Pickart, 1999; VanDemark et al., 2001; Eddins et al., 2006; Plans et al., 2006). Like many ubiquitin E3s, RNF8 can interact with multiple E2s, and preferential assembly of RNF8 with Ubc13–Mms2 at DNA repair centers is mediated by the HECT E3 ligase HERC2 (Bekker-Jensen and Mailand, 2010). Association of RNF8 with Ubc13–Mms2 does not appear to strictly require the catalytic activity of HERC2, but rather is promoted through interaction of HERC2 with RNF8, an interaction in turn regulated by phosphorylation and sumoylation of HERC2 (Bekker-Jensen and Mailand, 2010; Danielsen et al., 2012). HERC2 is a target of the SUMO E3 ligase PIAS4 (protein inhibitor of activated STAT protein 4), and also contains a novel ZZ zinc finger SUMO-binding domain (Danielsen et al., 2012). HERC2 is dependent on both sumoylation and its SUMO-binding domain for interaction with RNF8, suggesting that an intramolecular SUMO–SIM interaction may induce a conformational change in HERC2 to enable binding to RNF8, stabilizing RNF8–Ubc13 association (Danielsen et al., 2012).

PIAS4 also mediates sumoylation of RNF168, which may be important for maintaining sufficient RNF168 protein levels, since depletion of PIAS4 leads to decreased RNF168 half-life, and decreased transcript levels (Danielsen et al., 2012).



NEGATIVE REGULATION OF UBIQUITIN SIGNALING AT DNA BREAKS: SHIFTING THE BALANCE OF DNA DSB REPAIR PATHWAYS

Antagonizing the recruitment of UBD-containing repair proteins to sites of DNA damage is also an important regulatory mechanism in repair of DNA DSBs, and may function in shifting the balance between DNA DSB repair by NHEJ versus HR. Recent studies uncovered three distinct mechanisms for antagonizing ubiquitin-dependent protein recruitment to DSBs: (1) turnover of ubiquitin chains by DUBs, (2) ejection of ubiquitinated proteins by an ubiquitin-directed segregase, and (3) competition by an RNF168 paralog.

The NHEJ pathway is promoted by assembly of 53BP1 at DNA repair centers (Escribano-Diaz et al., 2013; Zimmermann et al., 2013). Formation of K63 ubiquitin chains on H2AX promotes recruitment of 53BP1 to repair foci through a currently unknown mechanism (Hartlerode et al., 2012). Efficient recruitment of 53BP1 to breaks is also dependent on interaction of the Tudor domains in 53BP1 with H4K20me2 (Figure 2). This constitutive H4 modification is enriched at breaks due to the H4 methyltransferase MMSET, which localizes to breaks following

damage (Pei et al., 2011). However, two proteins, JMJD2A and L3MBTL1, appear to have a common function in obstructing access of 53BP1 to histone H4 (Acs et al., 2011; Meerang et al., 2011; Mallette et al., 2012). Importantly, association of these proteins with chromatin is regulated by ubiquitination through two distinct pathways (Figure 2; Acs et al., 2011; Meerang et al., 2011; Mallette et al., 2012).

JMJD2A is a Tudor domain-containing protein that binds H4K20me2 with higher affinity than 53BP1 (Mallette et al., 2012). Accessibility of 53BP1 to H4K20me2 is enabled through proteasomal degradation of JMJD2A triggered by RNF8/RNF168-mediated K48-linked ubiquitin chains (Mallette et al., 2012). This study demonstrated that assembly of RNF8 with UbcH5c enables it to catalyze K48-linked chains, highlighting the importance of RNF8 in catalyzing both K48- and K63-linked ubiquitin at DNA repair centers.

L3MBTL1 is a Polycomb protein that binds H4K20me2 through multiple MBT domains, and is ejected from these sites by the “molecular corkscrew” activity (Ramadan, 2012) of the AAA-ATPase p97/VCP (valosin-containing protein; Acs et al., 2011; Meerang et al., 2011). The p97-UFD1-NLP4 complex has ubiquitin-dependent segregase activity, and requires RNF8 for turnover of K48-linked ubiquitin chains at DNA breaks (Acs et al., 2011; Meerang et al., 2011). One of the functions of this segregase activity is to displace L3MBTL1 from chromatin at DNA breaks, unmasking the binding site for 53BP1 (Acs et al., 2011; Meerang et al., 2011).

Attenuation of ubiquitin signaling at DNA breaks is also regulated by two members of the JAMM/MPN+ family of DUBs that specifically hydrolyze K63-linked ubiquitin chains: the BRCA1-A complex member BRCC36, and the 19S proteasomal lid subunit POH1 (Cooper et al., 2009; Shao et al., 2009; Butler et al., 2012). Cells deficient in BRCC36 or POH1 are sensitized to IR, implicating a role for proteolysis of K63 chains in the DDR (Shao et al., 2009; Butler et al., 2012). BRCC36 and POH1 antagonize the actions of RNF8/RNF168, hydrolyzing the K63 linkages that promote 53BP1 recruitment. POH1 promotes association of JMJD2A with chromatin, and therefore suppresses 53BP1 recruitment and the NHEJ pathway (Butler et al., 2012). POH1 also appears to promote HR by a mechanism independent of 53BP1 (Butler et al., 2012). In cells with deficient RNF8/RNF168 activity, formation of 53BP1 foci and NHEJ pathway utilization can be restored by co-depletion of POH1 (Butler et al., 2012).

Accumulation of K63 ubiquitin chains at DNA repair centers is also antagonized through RNF169-mediated competition with UBD-containing proteins for binding sites at DNA DSBs. Through bioinformatics analyses, three groups independently identified RNF169 as a paralog of RNF168, suggesting potential involvement of RNF169 in the DDR signaling cascade (Chen et al., 2012; Panier et al., 2012; Poulsen et al., 2012). Following DNA damage, RNF169 is targeted to repair foci through one of its two UBDs, MIU2 (Chen et al., 2012; Panier et al., 2012; Poulsen et al., 2012). RNF168 is also required for accumulation of RNF169 at repair foci (Chen et al., 2012; Panier et al., 2012; Poulsen et al., 2012). Although purified RNF169 displays E3 ligase activity, unlike RNF168 it is inactive toward H2A (Poulsen et al., 2012). Instead, RNF169 inhibits recruitment of proteins that

depend on RNF8/RNF168 activity for recruitment to repair foci. Over-expression of RNF169 out-competes RNF168 for association with chromatin, leading to a reduction in ubiquitinated proteins at breaks, and impairing 53BP1 accrual at DNA repair foci, causing a delayed DDR (Chen et al., 2012; Panier et al., 2012; Poulsen et al., 2012). Consistently, depletion of RNF169 leads to prolonged DDR signaling and a sustained G₂/M checkpoint after damage (Chen et al., 2012).

What is the functional significance of opposing RNF8/RNF168-dependent K63 ubiquitination? One emerging hypothesis is that K63 signaling mediates 53BP1 assembly at DNA breaks, promoting the NHEJ pathway (**Figure 1**), since knock-down of RNF168 selectively affects NHEJ (Meerang et al., 2011; Poulsen et al., 2012). 53BP1 and RAP80 seem to suppress HR-mediated repair (Bothmer et al., 2010; Coleman and Greenberg, 2011; Hu et al., 2011; Escribano-Diaz et al., 2013; Zimmermann et al., 2013); therefore, inhibiting their recruitment to DNA breaks may promote the HR pathway. In line with this hypothesis, depletion of RNF169 reduces HR repair, while over-expression of RNF169 causes increased HR efficiency (Poulsen et al., 2012). A shift toward HR-mediated repair may be favorable since it is less error-prone than the NHEJ pathway.

DNA DAMAGE-INDUCED SUMOYLATION OF 53BP1 AND BRCA1 BY PIAS1 AND PIAS4

Small ubiquitin-like modifiers and the SUMO conjugation machinery, the E1 SAE1 (SUMO Activating Enzyme E1) and the E2 Ubc9 localize to breaks following damage (Galanty et al., 2009; Morris et al., 2009). Recruitment of SUMO-1 and SUMO-2/3 to DNA breaks is dependent on the SUMO E3 ligases PIAS1 and PIAS4. PIAS1 is specifically required for SUMO-2/3 recruitment while PIAS4, and another SUMO E3 ligase, CBX4, promote recruitment of SUMO-1 and SUMO-2/3 (Galanty et al., 2009; Ismail et al., 2012). Depletion of either PIAS1 or PIAS4 impairs recruitment of BRCA1 (breast cancer 1) and RPA (replication protein A), while depletion of PIAS4 impairs recruitment of 53BP1 (Morris et al., 2009; Galanty et al., 2012). PIAS4 is required for sumoylation of 53BP1 following damage, though the function of this modification remains to be determined. PIAS1 and PIAS4 each promote sumoylation of BRCA1 at K119, which stimulates its ubiquitin ligase activity (Morris et al., 2009). Regulation of the two DSB repair pathways may be mediated by different isoforms of SUMO; depletion of 53BP1 impairs SUMO-1 but not SUMO-2/3 accumulation, while BRCA1 depletion impairs SUMO-2/3 but not SUMO-1 accumulation (Galanty et al., 2009).

RNF4: LINKING SUMOYLATION AND UBIQUITINATION IN THE DNA DAMAGE RESPONSE

The sumoylation and ubiquitination pathways are directly linked by the E3 RNF4, a member of the SUMO-targeted ubiquitin ligase (STUbl) family that, through four amino-terminal SIMs, preferentially binds and ubiquitinates poly-sumoylated proteins (Prudden et al., 2007; Perry et al., 2008; Tatham et al., 2008). RNF4 has an established role in the DDR, as RNF4 depletion causes increased IR signaling, and impairs RAP80, BRCA1, and RAD51 recruitment to sites of DNA damage (Guzzo et al., 2012; Luo

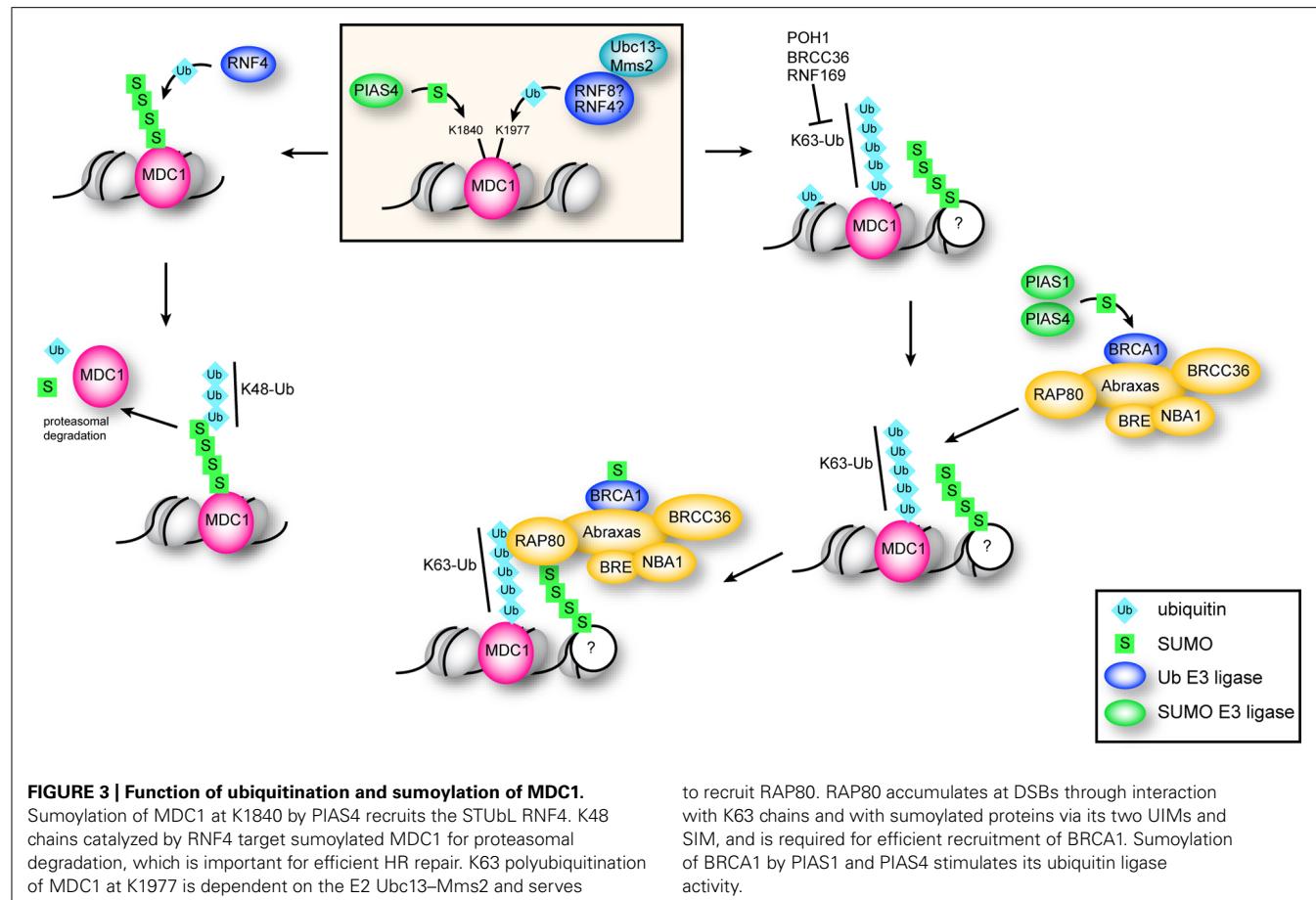
et al., 2012; Vyas et al., 2012; Yin et al., 2012). RNF4 ubiquitinates several sumoylated proteins in the DDR cascade, including MDC1, BRCA1, and RAP80 (Guzzo et al., 2012; Luo et al., 2012; Vyas et al., 2012), and is required for turnover of MDC1 and RPA (Galanty et al., 2012; Luo et al., 2012). Paired with the E2 UbcH5c, RNF4 can catalyze K11-, K48-, or K63-linked ubiquitin chains (Tatham et al., 2008), and at DNA breaks RNF4 contributes specifically to K48 (Luo et al., 2012) and K63 (Yin et al., 2012) linkages.

Although depletion of RNF4 affects both HR and NHEJ, RNF4-mediated ubiquitination of MDC1 specifically impacts the HR pathway of DNA DSB repair by preventing excess accumulation of MDC1 at repair foci (Luo et al., 2012). Following DNA damage, modification of K1840 of MDC1 by SUMO-2/3 recruits RNF4 to sites of DNA breaks (Luo et al., 2012; Yin et al., 2012). MDC1 is then targeted for proteasomal degradation via RNF4-mediated K48-linked ubiquitin chains (Luo et al., 2012; **Figure 3**). Since H2AX/MDC1/53BP1 retention at DNA breaks is antagonistic to HR (Bouwman et al., 2010; Bunting et al., 2010; Helmink et al., 2011), failure to sumoylate MDC1, for example by mutation of K1840, leads to its retention at DNA breaks and inhibition of HR (Luo et al., 2012). MDC1 also mediates recruitment of RAP80 to DNA damage foci. Although the E3 ligase remains unknown, ubiquitination of K1977 within the BRCT domain of MDC1 is required for recruitment of RAP80 to DNA DSBs (Strauss and Goldberg, 2011; Strauss et al., 2011). The K63-specific E2 Ubc13–Mms2 is required for RAP80 recruitment, implying that RAP80 is recruited to breaks through K63-linked ubiquitin chains (Strauss and Goldberg, 2011; Strauss et al., 2011). Specifically, RAP80 is targeted to ubiquitin–SUMO hybrid chains through its SIM and two UIMs, and mutation of either the UIMs or the SIM in RAP80 decreases RAP80 recruitment to repair centers (Guzzo et al., 2012; Hu et al., 2012).

RNF20–RNF40-MEDIATED H2B UBIQUITINATION: A CRITICAL ROLE IN DNA DSB REPAIR THROUGH CHROMATIN REMODELING

Another important histone modification in DNA repair is the monoubiquitination of histone H2B (ubH2B) on K120, which is an important modification associated with transcriptional elongation in undamaged cells (Xiao et al., 2005). This modification is also required for di-and tri-methylation of K4 and K79 of histone H3 at transcribed chromatin (Kim et al., 2009). At the structural level, ubH2B was shown to interfere with chromatin compaction, leading to an open, more accessible conformation (Fierz et al., 2011). Importantly, the alteration in chromatin structure observed was not simply due to the steric bulk of an added ubiquitin residue. Rather, it was due to intrinsic properties of the modification itself, although the exact residues involved in this chromatin restructuring have yet to be identified. This relaxed conformation may then enhance accessibility of underlying DNA to various transcription factors and their co-regulators.

The E3 ligase responsible for monoubiquitination of H2B is a tight heterodimeric complex of RING-finger proteins RNF20 and RNF40 (Kim et al., 2005; Zhu et al., 2005). Recently, the role of RNF20–RNF40-mediated H2B monoubiquitination in DNA DSB repair has been investigated in several studies (Chernikova



et al., 2010; Moyal et al., 2011; Nakamura et al., 2011). These studies demonstrate that monoubiquitination of H2B is required for timely break repair, as abrogation of ubH2B by either RNF20–RNF40 knock-down or over-expression of a non-ubiquitinatable H2B point mutant leads to increased sensitivity to DNA damaging agents with a subsequent reduction in DSB repair efficiency. In addition, when transcription inhibitors are used to reduce the effect of transcription-associated H2B ubiquitination, an elevation of ubH2B can be observed following the induction of DNA DSBs. Notably, a fraction of RNF20–RNF40 was also found to be recruited to DNA DSBs following DNA damage and to interact with ATM and NBS1 (Chernikova et al., 2010; Moyal et al., 2011). Not only does RNF20–RNF40 physically interact with ATM but it also undergoes ATM-mediated phosphorylation, which appears to be required for damage-induced H2B monoubiquitination. However, RNF20 depletion did not affect DNA damage-induced phosphorylation and ubiquitination of H2AX. In fact, several proteins that are recruited via γ-H2AX to break sites such as 53BP1, ATM and MDC1 still formed normal foci in RNF20-depleted cells (Nakamura et al., 2011) suggesting that the RNF20–RNF40 pathway functions independently and/or in parallel to the γ-H2AX-mediated DDR cascade. Collectively these studies demonstrated that ubH2B is not required for the recruitment of damage sensors in early stages of the DDR but is essential for the accumulation of DNA DSB repair proteins involved in both

NHEJ (XRCC4 and KU80) and HR (RAD51, RPA, and BRCA1) at DSBs. In addition, both NHEJ and HR repair pathways display retarded repair kinetics when H2B monoubiquitination is abrogated.

Due to the requirement of H2B monoubiquitination for H3K4 and H3K79 methylation during transcription, Moyal et al. (2011) and Nakamura et al. (2011) examined whether ubH2B-dependent methylation at these sites also occur in response to DSBs. While Moyal et al. (2011) did not observe significant differences in methylation, Nakamura et al. (2011) demonstrated that depletion of RNF20 significantly reduces H3K4 and H3K79 methylation following DSB induction. In addition, they noted that SNF2h (sucrose non-fermenting 2 homolog), a subunit of the ATP-dependent chromatin-remodeling complex ISWI that is recruited to sites of transcription through an interaction with methylated H3K4, is recruited to DSBs in an RNF20–RNF40-dependent manner. SNF2h depletion leads to reduced DSB repair through the HR pathway suggesting that chromatin-remodeling mediated by SNF2h influenced repair efficiency. To further support this notion, Nakamura et al. (2011) demonstrated that treatment with several agents that induced chromatin relaxation counteracts RNF20 defects in DNA DSB repair. These experiments suggest that monoubiquitination of H2B by RNF20–RNF40 facilitates chromatin decondensation, possibly through SNF2h-mediated chromatin remodeling, so that repair proteins

can access the underlying DNA. Since H3K79 methylation by DOT1L and binding of RAD9 via its Tudor domain is also required for efficient single-stranded DNA generation and HR (Lazzaro et al., 2008), changes in H3K79 methylation rather than chromatin-remodeling *per se* may be responsible for the observed defects in DNA DSB repair associated with depletion of RNF20.

CHROMATIN REMODELING-ASSISTED UBIQUITINATION IN THE DSB RESPONSE

As has been discussed above, ubiquitination can lead to chromatin structural rearrangements in response to DSBs. However, there is evidence that ubiquitin-independent chromatin-remodeling can also facilitate ubiquitination at DSBs, termed chromatin remodeling-assisted ubiquitination. For example, one study recently demonstrated a role for RNF8 in DNA repair that does not depend on its catalytic activity (Luijsterburg et al., 2012). RNF8 was found to recruit the ATPase CHD4 of the nucleosome-remodeling and deacetylase (NuRD) chromatin-remodeling complex to DNA repair foci, rendering DNA more amenable to ubiquitination (Denslow and Wade, 2007; Luijsterburg et al., 2012). Lack of CHD4 activity led to decreased ubiquitination at DSBs and consequently, defective BRCA1 recruitment (Luijsterburg et al., 2012). The authors demonstrate that CHD4 is required for efficient ubiquitination of chromatin, as RNF8 is only briefly associated with chromatin (Mailand et al., 2007) and artificially prolonging RNF8 retention at chromatin bypassed the need for CHD4. The authors propose that RNF8-mediated CHD4 recruitment, and subsequent chromatin decondensation could create a more amenable local chromatin environment for ubiquitination by promoting RNF168 and BRCA1 assembly.

Another study describes a role for the p400 ATPase (a component of the mammalian NuA4 complex) in regulating nucleosome stability and RNF8-mediated chromatin ubiquitination in DNA DSB repair (Xu et al., 2010). DNA damage destabilizes nucleosomes within chromatin regions surrounding DNA DSBs in an active process requiring the ATPase activity of p400, in addition to the histone acetylation activity of the acetyltransferase Tip60. p400 was found to be recruited to DNA DSBs through interaction with MDC1, which was independent of ATM phosphorylation. Interestingly, suppression of RNF8 did not affect the p400-mediated decrease in nucleosome stability at DNA DSBs, indicating that RNF8 ubiquitination does not contribute to p400 chromatin-remodeling activity. However, RNF8-dependent ubiquitination and the subsequent recruitment of BRCA1 and 53BP1 at DNA DSBs required nucleosome destabilization by p400. The authors propose a model whereby DSB induction leads to the generation of γ-H2AX and subsequently the recruitment of MDC1. Components of the NuA4 complex, importantly p400 and Tip60 are recruited to breaks through MDC1, and the ATPase activity of p400 in conjunction with Tip60 histone acetylation then disrupts local chromatin structure leading to a more open, relaxed conformation. This open conformation exposes RNF8 ubiquitination targets as well as histone methylation sites such as H4K20me2, facilitating recruitment of PIAS1/PIAS4, BRCA1, and 53BP1 to DNA DSBs.

Here we have described two different instances of chromatin remodeling-assisted ubiquitination involving RNF8: one involving CHD4 of the NuRD complex, and the other the ATPase p400 of NuA4. It is clear that multiple chromatin-remodeling events take place in response to DNA DSBs. Whether they all function simultaneously or are evoked in response to different stimuli to mediate alternative repair pathways (NHEJ or HR for instance) remains to be determined. Deciphering the exact mechanism involved in DNA DSB-induced chromatin restructuring represents a challenge for future studies.

SUMOYLATION OF THE KRAB DOMAIN-ASSOCIATED PROTEIN 1 AND THE REPAIR OF DNA BREAKS WITHIN HETEROCHROMATIN

Post-translational modification of many transcription factors or cofactors by sumoylation is generally associated with transcriptional repression (Verger et al., 2003). SUMO modification provides binding sites for diverse chromatin-remodeling enzymes and chromatin-associated proteins such as histone deacetylase 2 (HDAC2), histone demethylase LSD1, heterochromatin protein 1 (HP1), and the NuRD complex that subsequently mediate chromatin compaction and gene silencing (Ouyang and Gill, 2009).

Sumoylation of the transcriptional co-repressor KAP1 (KRAB domain-associated protein 1) is involved in the maintenance of heterochromatin structure. KAP1 is an SUMO E3 ligase, which undergoes auto-sumoylation (Ivanov et al., 2007) and directly interacts with the NuRD complex (Schultz et al., 2001), promoting ATP-dependent chromatin compaction in heterochromatin. NuRD is a multi-subunit complex that couples ATPase chromatin-remodeling activities (through Mi-2 proteins CHD3 and CHD4) with histone deacetylation (through HDAC1/HDAC2 subunits; Goodarzi et al., 2011). The interaction between KAP1 and the NuRD complex is mediated by the CHD3 component, which contains a SIM at its carboxy-terminus. Due to its role in chromatin compaction, KAP1 poses a substantial barrier to DNA DSB repair in heterochromatin. In order for effective repair to occur within heterochromatin, dynamic alterations to chromatin structure are required. Phosphorylation of KAP1 (pKAP1) on S824 by ATM has been shown to be essential for DSB repair in heterochromatic regions (Goodarzi et al., 2008), and to enhance cellular survival following IR (Ziv et al., 2006; Noon et al., 2010).

A recent study put forth a mechanism of pKAP1-mediated chromatin relaxation and heterochromatic DSB repair (Goodarzi et al., 2011). Following IR, ATM induces pKAP1, resulting in dispersion of CHD3 from DNA DSBs, and also triggering a relaxation of chromatin structure. Importantly, CHD3 depletion alleviated repair defects caused by inhibition of ATM or the expression of a non-phosphorylatable S824A KAP1 mutant. CHD3 activity is targeted to KAP1 through interactions between its SIM domain and sumoylated KAP1, and consequently ablation of this interaction by expression of KAP1 with mutated SUMO conjugation sites bypasses the role of pKAP1 in repair. Collectively this data suggests that CHD3 activity associated with sumoylated KAP1 is inhibitory to DSB repair; however, this effect can be alleviated by ATM-mediated pKAP1.

Two possible scenarios can be envisaged for how CHD3 mediates chromatin structural changes in KAP1-dependent heterochromatin following DNA damage. First, CHD3 activity could affect sumoylated KAP1 levels; however, levels of KAP1-SUMO are not altered upon DSB induction. Alternatively, DSB-induced pKAP1 might directly interfere with the interaction between CHD3 and KAP1-SUMO. Consistent with this theory, reduced amounts of CHD3 were observed to interact with phosphomimetic KAP1 following IR. Goodarzi et al. (2011) postulate that DNA damage-induced pKAP1 increases negative charge at the carboxy-terminal region of KAP1, effectively interfering with interactions between SUMO conjugated to KAP1 and the SIM domain of CHD3. This would result in the release of CHD3 from KAP1-enriched heterochromatin to relax chromatin structure and facilitate DSB repair.

The regulated dephosphorylation of KAP1 may also play a parallel or additive role in regulating heterochromatin organization during the DDR (Li et al., 2007, 2010). For example, dephosphorylation of pKAP1 at Ser824 by protein phosphatase 1 (PP1) was shown to regulate sumoylation of KAP1 (Li et al., 2010). Importantly, two PP1 isoforms (PP1 α and PP1 β) were found to differentially interact with KAP1 (PP1 α under unstressed conditions and PP1 β under genotoxic stress) and to dephosphorylate KAP1 at Ser824. PP1 α was found to regulate basal KAP1 dephosphorylation while PP1 β played a role in dephosphorylation of KAP1 Ser824 following modification by ATM kinase in response to DNA DSBs. It was postulated that PP1 α , which is constitutively associated with KAP1, may serve to set a threshold for the degree of ATM pKAP1 required to overcome S824 dephosphorylation and consequently sumoylation of KAP1 during the DDR. In this model, after DNA repair is complete, PP1 α in conjunction with PP1 β would then serve to restore KAP1 sumoylation levels, and hence its role in transcriptional repression and the maintenance of heterochromatin structure.

CONCLUSION AND FUTURE DIRECTIONS

The study of the regulation of the cellular response to DNA damage is a rapidly advancing field. Findings from the last few years have underscored a role for ubiquitin and SUMO in the DDR signaling cascade. While many substrates of sumoylation and ubiquitination have been identified, for many of these target proteins the modification sites have yet to be determined. The next stage in our understanding of the DDR will require identification of individual modification sites in these proteins in order to assign specific functions to each sumoylation and ubiquitination event. Abolishing sumoylation of a single protein in a DDR pathway may not, however, always yield appreciable phenotypes. For example, one recent study demonstrated that in yeast, simultaneous mutation of the sumoylation sites in multiple repair proteins was required to significantly affect the repair of DNA DSBs by the HR pathway (Psakhye and Jentsch, 2012). This study hints to the potential for a high degree of redundancy in the signaling pathways employing UBLs for the regulation of the DDR, with the caveat that the universality of these results cannot be determined until similar studies are completed in other organisms. In addition, other UBLs, such as NEDD8 and ISG15, have also been implicated in

the DDR (Desai et al., 2008; Jeon et al., 2012; Blank et al., 2013), which implies a similarly complex networks of E3 ligases and substrates for these UBLs may also exist as a means of controlling the DDR. Therefore, future studies should be directed to investigating the potential role of these other UBL proteins in the DDR, which will further add to our understanding of regulatory post-translational modification networks in the cellular response to DNA DSBs.

There are several gaps in our current understanding of ubiquitin signaling at DNA breaks. For example, the mechanism through which monoubiquitination of H2AX by RNF2 leads to recruitment of ATM has not been elucidated. As highlighted by Mattioli et al. (2012), the dependence of RNF168 recruitment on the catalytic activity of RNF8 has not yet been explained; therefore, further studies should pursue identification of additional RNF8 substrates. While we now have an understanding of how ubiquitin regulates ejection of JMJD2A and L3MBTL1 from the chromatin docking sites for 53BP1, the extent of overlap of these two pathways is not clear. As well, the predicted UBDs and newly identified SIMs in several members of the BRCA1-A complex (Abraxas, BRCC36, BRE; Guzzo et al., 2012) will need to be assessed for their potential contribution to DNA repair.

In addition, there is mounting evidence that the two major sites of SUMO-1 and SUMO-2/3 accumulation in the cell, the nuclear lamina and promyelocytic leukemia nuclear bodies (PML NBs), may play diverse roles in the DDR. Both of these compartments are enriched in SUMO E3 ligases and STUBLs, including RanBP1 and Slx5–Slx8 (the yeast RNF4 homolog) at the nuclear lamina and PIAS1, PIAS4, and RNF4 in PML NBs (Pichler et al., 2002; Nagai et al., 2011). In particular, it should be noted that PML is sumoylated, contains a SIM, and was one of the first identified substrates of RNF4, which regulates PML degradation in response to arsenic treatment (Dellaire and Bazett-Jones, 2004; Bernardi and Pandolfi, 2007; Lallemand-Breitenbach et al., 2008; Tatham et al., 2008). PML NB number is also regulated by DNA damage through ATM and KAP1 (Dellaire et al., 2006; Kepkay et al., 2011), and these bodies are associated with a host of DNA repair factors and cell cycle checkpoint proteins that shuttle to and from this subnuclear domain in response to DNA DSBs; these include BLM, WRN, NBS1, MRE11, TopBP1, CHK2, and p53, several of which are targets of sumoylation themselves (Dellaire and Bazett-Jones, 2004). Finally, both of these compartments are also associated with “late” DNA repair foci that may indicate unrepaired or difficult to repair DNA DSBs in mammalian cells (Dellaire et al., 2009). In yeast, unrepaired breaks are recruited to the nuclear lamina where they are sequestered as a possible means of inhibiting inappropriate HR (Oza et al., 2009; Lisby et al., 2010) whereas the juxtaposition of DNA breaks at PML NBs in mammalian cells may enhance HR, as depletion of PML impairs the HR pathway of DNA repair (Yeung et al., 2012). Given the multi-faceted association of these compartments with both DNA repair processes and the sumoylation machinery, future studies should look beyond DNA repair foci to consider the role of PML NBs and the nuclear lamina in coordinating the trafficking, post-translational modification and degradation of proteins in the DDR that are subjected to modification by UBLs.

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It takes two to tango: Ubiquitin and SUMO in the DNA damage response

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The complexity of living cells is primarily determined by the genetic information encoded in DNA and gets fully disclosed upon translation. A major determinant of complexity is the reversible post-translational modification (PTM) of proteins, which generates variants displaying distinct biological properties such as subcellular localization, enzymatic activity and the ability to assemble in complexes. Decades of work on phosphorylation have unambiguously proven this concept. In recent years, the covalent attachment of Ubiquitin or Small Ubiquitin-like Modifiers (SUMO) to amino acid residues of target proteins has been recognized as another crucial PTM, re-directing protein fate and protein-protein interactions. This review focuses on the role of ubiquitylation and sumoylation in the control of DNA damage response proteins. To lay the ground, we begin with a description of ubiquitylation and sumoylation, providing established examples of DNA damage response elements that are controlled through these PTMs. We then examine in detail the role of PTMs in the cellular response to DNA double-strand breaks illustrating hierarchy, cross-talk, synergism or antagonism between phosphorylation, ubiquitylation and sumoylation. We conclude offering a perspective on Ubiquitin and SUMO pathways as targets in cancer therapy.

Keywords: ubiquitylation, sumoylation, phosphorylation, DNA damage response, cancer therapy

INTRODUCTION

The components of signal transduction pathways are organized in a hierarchical manner and communicate with one another. In its simplest formulation, a signaling pathway can be represented with a linear cascade where unidirectional arrows connect a stimulus to the final response through a defined number of intermediates. The recent sequencing of animal and plant genomes and the advent of systems biology have changed this perspective. Proteome scale interaction studies have unveiled the existence of interfaces between pathways and shown that the multiplicity of interactions among their components likely accounts for the array of outputs observed in biological systems. While this novel perspective represented *per se* a step forward, it still had the intrinsic limitation of merely providing a static snapshot of biological networks. The need for a more realistic picture of signal transduction prompted the development of predictive modeling that, by representing the dynamic flow of information, accounts for the fluctuation of variables as it actually occurs in defined biological systems (Barabasi and Oltvai, 2004). Despite their intrinsic limitation though, “snapshots” provided by reductionist approaches currently represent our best option to study and explain the functioning of signal transduction networks at the molecular level.

Considering that proteins are the constitutive elements of cellular networks and they hierarchically relate to each other, modification of structural or enzymatic traits of one or more elements in a network will necessarily affect network properties and result in outputs that are directly observable (i.e., cell proliferation in response to growth factors, cell cycle arrest or terminal

differentiation in response to antimitogens or differentiation factors, respectively). Alteration of the properties of network components is achieved through post-translational modifications (PTM), consisting in the covalent addition of chemical groups to one or more amino acids of a protein target in a manner that is, in most cases, reversible. The hierarchical, synergistic or antagonistic combination of PTMs defines a code that translates into distinct outputs.

HISTORICAL PERSPECTIVE

Ubiquitin entered the arena of scientific discoveries in the mid-seventies as result of serendipity and pioneering work initiated in the midst of more trendy studies addressing how the information contained in DNA is decoded to generate the variety of proteins that make up a cell (Ciechanover, 2009). Studies aimed at elucidating the molecular mechanism of liver regeneration led to the identification of a non-histone chromosomal protein, named A24, displaying physicochemical properties similar to those of histones. The localization of A24 in nuclear and nucleolar chromatin as well as its marked decrease upon nucleolar hypertrophy led to the suggestion that A24 might represent a rDNA repressor (Goldknopf et al., 1975). Ciechanover and colleagues came to the discovery of Ubiquitin from another front. Based on the concept that synthesis and destruction of cellular proteins are homeostatic, with a perfect equilibrium being a necessary condition for life, they undertook studies on mechanisms of protein degradation. Using reticulocytes as model system, that are known to get rid of lysosomes during terminal differentiation but retain the ability of degrading hemoglobin,

they set out to identify the non-lysosomal mechanism of protein degradation present in these cells. Using classic biochemical protocols consisting of chromatographic fractionation of crude cell extracts followed by reconstitution of the enzymatic activity of interest through complementation of fractions, they discovered that proteolysis occurs through a cascade of events culminating in the covalent addition of a heat-stable component to proteins targets. Such component was named ATP-dependent proteolysis factor 1 (APF-1) and is now known as Ubiquitin (Ciehanover et al., 1978). Protein modification by APF-1, in turn, was shown to facilitate selective target recognition by the proteolytic machinery (Hershko et al., 1980). The subsequent discovery of several Ubiquitin-like proteins (UBLs) helped shedding light on the complexity of this PTM. UBLs were essentially demonstrated to have functions other than the control of protein degradation. This is the case of the “Small Ubiquitin-like Modifier”, in short SUMO, which was identified as a PTM of RanGAP (Matunis et al., 1996; Mahajan et al., 1997), the activator of the GTPase Ran that controls shuttling of cargos across the nuclear membrane. Sumoylation was shown to facilitate association of RanGAP with the nuclear envelope (Mahajan et al., 1998). Other notable examples are NEDD8, which can be covalently linked to cullins (Hori et al., 1999), the scaffold components of multisubunit Ubiquitin E3-ligases, in a manner that affects their activity; ISG15, which is conjugated to target proteins upon IFN α/β -induced viral response or inflammation (Jeon et al., 2010; Zhao et al., 2013); Urm1, which has low sequence homology to Ubiquitin (Goehring et al., 2003), though it displays a similar fold and is involved in oxidative stress responses in yeast; and, finally, the Atg cascade controlling autophagy in yeast and man, which is the main mechanism responsible for the degradation of cellular components in response to nutrients starvation. This consists of the E1-like enzyme Atg7, the E2-like components Atg3 and Atg7, and the E3-like Atg12-Atg5 conjugate that facilitates transfer of the Ubiquitin-like modifier Atg8 to phospholipids (Hanada et al., 2007).

UBIQUITYLATION

Ubiquitin is a highly conserved regulatory protein of 76 amino acids (8.5 kDa), which is constitutively expressed in all tissues of eukaryotic organisms. In mammalian cells, Ubiquitin is encoded by 4 genes: RSP27A, UBA52, UBB, and UBC (Kimura and Tanaka, 2010). The ATP-dependent conjugation of Ubiquitin C-terminal glycine (G₇₆) to lysine residues in the substrate leads to the formation of an isopeptide bond. Ubiquitin itself contains seven lysines behaving as acceptors for additional Ubiquitin molecules to generate poly-chains. Ubiquitylation is carried out in a cascade of reactions: first, a thioester bond is formed in an ATP-dependent manner between a cysteine in the active site of the E1-activating enzyme and Ubiquitin G₇₆. Second, Ubiquitin is transferred to the active cysteine of an E2-conjugating enzyme. Finally, an E3-ligase enzyme binds the E2-Ub complex and transfers Ubiquitin to lysine residues of the acceptor substrate (Hershko and Ciechanover, 1998) (**Figure 1**). Mammalian cells express only 2 E1s, approximately 38 E2s and more than 600 E3s.

E2-CONJUGATING ENZYMES

E2-conjugating enzymes can be classified in 17 subfamilies (Michelle et al., 2009) characterized by an active core called UBC (Ubiquitin-conjugating) domain. Ubiquitin E2 enzymes are structurally similar to UBL modifiers E2s, though the former can specifically interact with the two E1s involved in ubiquitylation (Ye and Rape, 2009). Each E2 enzyme can interact with multiple E3s, as demonstrated for Cdc34 (E2) and SCF complexes (E3) (Skowyra et al., 1997) or the UBE2C/UBE2S (E2s) and the APC/C (anaphase promoting complex/cyclosome; E3) (Williamson et al., 2009) or as shown in network interaction studies (Markson et al., 2009). Specificity is provided by the N-terminal region of the E2 where the amino acidic sequence determines the secondary structure of loops (L1 and L2) that contact two loops and an α -helix of the E3 (Zheng et al., 2000). For E2s interacting with more than one E3, the residue involved in recognition usually differs from one E3 to the other (Zhang et al., 2005a). The binding affinity between Ubiquitin-charged E2s and their cognate E3s is generally high, rendering very fast the kinetic of interaction (Das et al., 2009). Moreover, binding sites for E1 and the specific E3 often overlap in the E2, such that the E2 must dissociate from the E3 to be charged with Ubiquitin by the E1 and vice-versa (Eletr et al., 2005). E2 enzymes catalyze Ubiquitin chains initiation and elongation. Whereas some of them, such as UBE2W and UBE2E in humans, are specifically used by their E3 BRCA1 for chain initiation, the heterodimeric complex UBE2N-UBE2V1 and UBE2K are mainly involved in chain elongation (Christensen et al., 2007; Rodrigo-Brenni and Morgan, 2007; Jin et al., 2008b). Few E2s can mediate both processes, as illustrated by yeast Cdc34 that, together with SCF, is responsible for initiating Ubiquitin chains formation on Sic1 (cell cycle inhibitor subunit of cyclin-dependent kinase 1) in a non-interacting manner and for chain elongation by direct interaction with the substrate (Petroski and Deshaies, 2005).

E3-LIGASES

E3s are often part of multimeric complexes and can be divided in two main classes: HECT (Homologous to E6AP COOH-terminus) and RING (Really Interesting New Gene). A cysteine in HECT E3s catalytic domain binds Ubiquitin and transfers it to the substrate in an E2-independent manner (Kulathu and Komander, 2012) (**Figure 1**). The C-terminal domain of HECT E3s is highly conserved and retains both catalytic activity and the determinants for chain type specificity (You and Pickart, 2001), while the N-terminal region determines substrate specificity (Huang et al., 1999). Established members of the HECT family are E6AP, a partner of the oncogenic E6 protein of human papillomavirus, responsible for p53 downregulation (Huang et al., 1999), Itch/AIP4, with roles in the inflammatory signaling pathways (Chastagner et al., 2006) and Nedd4 and Nedd4L that participate in the development of mouse central nervous system (Kumar et al., 1992).

The vast majority of E3-ligases known to date belongs to the RING family and is characterized by the presence of the Cys/His-rich RING finger domain. The RING finger brings in close proximity substrate and activated E2 enzyme, with the latter directly transferring Ubiquitin to the former (**Figure 1**).

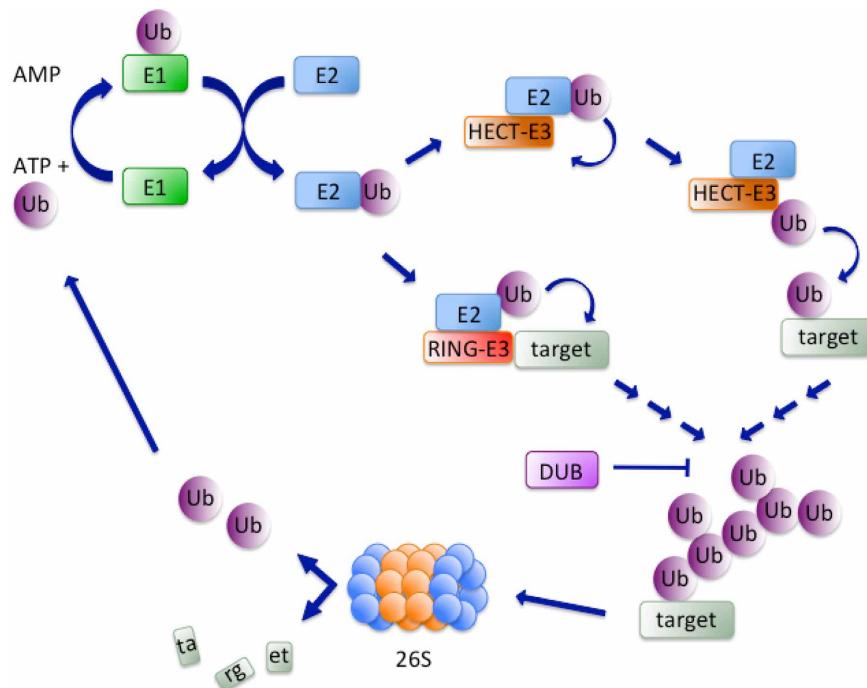


FIGURE 1 | Simplified scheme of ubiquitylation. The ubiquitylation cascade initiates with an ATP-dependent reaction consisting in the formation of a thioester bond between a cysteine in the active site of the E1-activating enzyme and G₇₆ in Ubiquitin (Ub). Next, Ubiquitin is transferred to the active cysteine of an E2-conjugating enzyme that interacts with an E3-ligase. The latter can either directly transfer

Ubiquitin to lysine residues of the acceptor substrate, as is the case for HECT-E3s, or recruit substrates to the E2 enzyme, a mechanism that characterizes RING-E3s. Finally, ubiquitylated substrates are shuttled to the 26S proteasome and Ubiquitin is recycled for another round of reactions. DUBs oppose substrate degradation by reversing the process of ubiquitylation.

A domain structurally related to the RING finger, the U-box, is found in many E3 ligases of this class (Deshaises and Joazeiro, 2009). Rad18 was the first identified RING domain-containing protein and, together with the E2-conjugating enzyme Rad6, was shown to be essential for post-replicative bypass of UV-induced DNA damage in yeast (Bailly et al., 1997). The RING domain, along with a B-box (zinc-binding fold similar to the RING) and a coiled-coil region (CC), collectively called RBCC supradomain, characterizes the 80 members of the TRIM (Tripartite Interaction Motif) family of E3-ligases (Marin, 2012). A small subfamily of E3 ligases is characterized by the presence of three RING domains: an N-terminal (N-RING), a in-between (IBR) and a C-terminal (RING2 or C-RING) (Eisenhaber et al., 2007). Parkin, a protein involved in Parkinson's disease, is the best-characterized member of this subfamily (Chaugule et al., 2011). The Cullin/RING Ubiquitin ligase (CRL) subfamily represents the largest subgroup of the RING-finger E3 ligases (Deshaises and Joazeiro, 2009). CRLs are multisubunit E3s composed of a RING finger domain protein (Rbx1 or Rbx2) responsible for recruiting the ubiquitylated E2 enzyme, a scaffold protein member of the Cullin family and a receptor for substrate recognition (F-box protein). Some CRLs additionally feature a linker protein, such as Skp1 in the SCF complex or CRL1 and DDB1 in the CRL4 complex (Deshaises and Joazeiro, 2009). CRLs are activated by a PTM consisting in conjugation of NEDD8 to the Cullin component (Pan et al., 2004).

ATYPICAL UBIQUITIN CHAINS

Ubiquitylation indicates the process of single Ubiquitin moiety addition to a substrate as well as its extension to form Ubiquitin polymers. Chain extension can occur at all seven lysine residues present on Ubiquitin (K₆, K₁₁, K₂₇, K₂₉, K₃₃, K₄₈, K₆₃) (Ikeda and Dikic, 2008). E2s such as UBE2N (UBC13) or UBE2RI (CDC34) show specificity for linkage to K₆₃ or K₄₈, respectively (Vandemark et al., 2001; Petroski and Deshaises, 2005). Others, like UBE2D and UBE2E, can promote different types of Ubiquitin chains formation (Kim et al., 2007). K₄₈ and K₆₃ linked chains represent the two mostly studied modifications by Ubiquitin, with the first essentially involved in degradation by the 26S proteasome (Komander and Rape, 2012) and the second mainly affecting the function of signaling components (Sun and Chen, 2004) and DNA repair proteins (Chen and Sun, 2009). Proteins undergoing degradation are recognized by the substrate receptor component of the 26S proteasome only if they contain chains longer than four Ubiquitin moieties (Thrower et al., 2000). The processivity of such chains, which is the number of Ubiquitin moieties attached to a protein or to a growing Ubiquitin chain while it is associated to the E3 ligase, determines the rate of substrate degradation (Rape et al., 2006). K₆-linked chains do not likely have a proteolytic role (Kim et al., 2011). K₁₁-linkage, on the contrary, plays a key role in the degradation of cell cycle regulators as well as in endoplasmic reticulum associated degradation (ERAD) and membrane trafficking (Behrends and Harper, 2011).

Little is known about the relatively low abundant K₂₇-, K₂₉- and K₃₃-linkages (Komander and Rape, 2012). An additional type of Ubiquitin chain assembly was recently discovered, the so-called LUBAC (Linear Ubiquitin Chains Assembly Complex), which is formed by a complex of two E3 RING-finger ligases, HOIL-1L and HOIP. This type of linkage is characterized by head-to-tail assembly, in which the C-terminal glycine of the previous Ubiquitin is linked to the methionine residue of the next Ubiquitin. Linearly-linked Ubiquitin chains are mainly involved in targeting proteins with a role in innate and adaptive immune signaling pathways (Walczak et al., 2012). Finally, evidence for the presence of more than one linkage type in the same poly-Ubiquitin chain has been provided (Kim et al., 2007). Mixed Ubiquitin chains were shown to result from the activity of the RING finger proteins Ring1B and Bmi1. The latter are components of the Polycomb repressive complex 1 (PRC1), with Ring1B displaying E3-ligase activity toward histone H2A. Mono-ubiquitylation of H2A was shown to depend, at least *in vitro*, from self-ubiquitylation of Ring1B through the generation of atypical mixed K6-, K27-, and K48-based chains on the same Ubiquitin molecule (Ben-Saadon et al., 2006).

DEUBIQUITYLATING ENZYMES

Ubiquitylation is a reversible process, with deUbiquitinases (DUBs) being responsible for the disassembly of Ubiquitin chains (Nijman et al., 2005). Deubiquitylation controls cell cycle transitions, proteasome- and lysosome-dependent degradation pathways, DNA repair, endocytosis and signal transduction pathways among others. Importantly, DUBs participate in controlling the dynamic state of histone ubiquitylation. An essential function played by DUBs is the co-translational activation of Ubiquitin, which is expressed as fusion to ribosomal proteins or in linear poly-Ubiquitin chains (Reyes-Turcu et al., 2009). A second important function is the recycling of free Ubiquitin from unattached chains (Komander et al., 2009). The human genome encodes approximately 100 DUBs, distinguished in five families: Ubiquitin C-terminal hydrolases (UCH), Ubiquitin specific proteases (USP/UBP), ovarian tumor (OUT), Josephines and JAB1/MPN/Mov34 metalloenzymes (JAMM) (Reyes-Turcu et al., 2009). Whereas the first four families behave as cysteine proteases, JAMM function as zinc-dependent metalloproteases. To prevent inappropriate or unscheduled cleavage of substrates, DUBs activity is controlled by a variety of PTMs, including phosphorylation, ubiquitylation and sumoylation (Reyes-Turcu et al., 2009). Besides the catalytic domain, DUBs feature protein-protein interaction domains and Ubiquitin-binding domains that facilitate formation of multimeric complexes and interaction with substrates, respectively. In most cases, binding to Ubiquitin causes DUBs to undergo conformational changes that expose the catalytic site, which is often hidden by a loop or a larger domain (Reyes-Turcu et al., 2009). DUBs such as USP14, UCH37, and POH1 are often found associated with the 19S subunit of the proteasome, a feature that allows hydrolyzing the poly-Ubiquitin chain from the substrate and recycling Ubiquitin prior to channeling the target protein into the proteasome (Finley, 2009). Reactive oxygen species (ROS) reversibly inactivate Cys-based DUBs, as exemplified by the key regulator of genomic

stability USP1, the oxidation of which facilitates PCNA mono-ubiquitylation and the consecutive recruitment of Pol η for the repair of oxidation-induced lesions (Cotto-Rios et al., 2012).

SHUTTLING TO THE PROTEASOME

The destiny of proteins modified by K₄₈ poly-Ubiquitin chains is degradation by the 26S proteasome. In the DNA damage response, this task is facilitated by shuttling orchestrated by dedicated receptor proteins such as yeast Rad23, Dsk2, Ddi1, and the Shp1/Cdc48/p97 complex. Receptor proteins recognize poly-Ubiquitin chains in their targets by virtue of Ubiquitin-Associated (UBA) domains and interact with subunits of the proteasome via Ubiquitin-Like (UBL) folds, thus effectively shuttling cargoes to the proteasome (Grabbe and Dikic, 2009). The yeast Rad23, which was originally identified for its role in nucleotide excision repair (NER), and its human homologues hHR23A and hHR23B are paradigmatic to this pathway. Rad23 contains two UBA and an N-terminal UBL domain that dynamically interacts with either one of the two UBA domains (Goh et al., 2008). Binding of an UBA domain to poly-Ubiquitin chains of the cargo protein displaces the UBL domain that becomes available for interacting with the proteasomal subunit 5a (Mueller and Feigon, 2003), facilitating the delivery of cargos to the proteasome. Paradigmatic is human p97 and its Ubiquitin-binding partner, the heterodimer UFD1-NPL4, that are recruited to DNA lesions and selectively remove K₄₈-Ubiquitin conjugates allowing the subsequent deposition of 53BP1, BRCA1, and Rad51 to regions undergoing repair (Meerang et al., 2011).

SUMOYLATION

SUMO proteins and Ubiquitin have only limited sequence identity but they fold in a similar manner (Bayer et al., 1998). SUMO-2 and SUMO-3 are 95% identical but display only 43% identity to SUMO-1. SUMO proteins are generated as inactive precursors and processed by Sentrin/SUMO-specific proteases (SENPs) that catalyze the removal of a C-terminal oligopeptide, exposing the glycine that is conjugated to lysine residues in the target (Xu and Au, 2005).

SUMO CASCADE

As for Ubiquitin, SUMO-1, SUMO-2, and SUMO-3 are conjugated to substrates through a dedicated E1-E2-E3 cascade. SUMO proteins bind the activating enzyme E1 [SAE1 and SAE2 in mammals, (Gong et al., 1999)] in an ATP-dependent manner and are transferred to the conjugating enzyme UBC9, which is the only E2 dedicated to SUMO conjugation (Johnson and Blobel, 1997). UBC9 is able to recognize and transfer SUMO to targets in the absence of a co-adjuvanting E3, though E3-like proteins containing an SP-RING domain facilitate the process by enhancing the affinity of UBC9 for its substrates (Bernier-Villamor et al., 2002). In the absence of an E3, acetylation apparently provides a means for UBC9 to discern between substrates carrying extended vs. regular recognition motifs (see below) (Hsieh et al., 2013).

The distinct mechanism of SUMO recognition and conjugation likely depends on the different distribution of charged residues on the surface of SUMO proteins as compared to Ubiquitin (Melchior, 2000). Of the SUMO E3-ligases identified to

date, some display exquisite specificity, such as RanBP2 that selectively targets RanGAP1 and Sp100 (Pichler et al., 2002). Others, like the PIAS family of proteins that are the mammalian homologues of yeast Siz proteins, act as repressors of STAT3 (Chung et al., 1997) and a number of transcription factors (Schmidt and Muller, 2003). Similarly to RING Ubiquitin ligases, the Siz/PIAS SUMO E3-ligases do not physically bind SUMO but rather interact non-covalently with it. Furthermore, through their zinc-binding SP-RING domain they associate with UBC9. In this manner Siz/PIAS bring SUMO-loaded UBC9 in close proximity to the protein target and facilitate transfer of the SUMO moiety (Hochstrasser, 2001). Among other SP-RING type SUMO E3s, TOPORS was the first reported example of an E3 ligase supporting the transfer of both Ubiquitin and SUMO (Rajendra et al., 2004; Weger et al., 2005).

SUMO CHAINS

SUMO-2 and SUMO-3 can polymerize to form chains on protein substrates whereas SUMO-1 is only added as monomer (Tatham et al., 2001). It is established that some substrates are modified either by SUMO1, namely RanGAP1 (Saitoh and Hinchey, 2000), or SUMO2/3, namely PML, whereas others are modified indifferently by both SUMO1 and SUMO2/3 (Vertegaal et al., 2006). The reason for such heterogeneity in the SUMO conjugation process is currently unknown, though it may be in part explained by the different pools of SUMO proteins available in the cell, with SUMO1 being mostly conjugated and SUMO2/3 forming a free pool that is mobilized in response to environmental stress (Saitoh and Hinchey, 2000).

The minimal core consensus sequence for recognition and sumoylation of target proteins is defined as Φ -K-X-D/E (with Φ being a hydrophobic residue). An extended sumoylation motif consisting in the sequence Φ -K-X-D/E-X₂-(E/D)₄₋₅ may comprise sites of phosphorylation in the acidic stretch that follows the sumoylated lysine (Yang et al., 2006).

The assembly of proteins complexes in response to sumoylation was addressed by means of two-hybrid screens that led to the discovery of proteins bearing SUMO-interacting motifs (SIMs) (Hannich et al., 2005).

DESUMOYLATING ENZYMES

As for other PTMs, sumoylation is a reversible process. The enzymes reversing sumoylation belong to the class of SENP proteins that control SUMO maturation from precursor polypeptides. Of the six SENP enzymes present in the mammalian genome, SENP1 and SENP2 display the ability of their yeast counterpart Ulp1 to control both the maturation of SUMO proteins and desumoylation reactions. SENP1 and SENP2 display a slight preference for pre-SUMO1 or pre-SUMO2/3, respectively, in the process of maturation but act equally well on both during deconjugation (Xu and Au, 2005). SENP3 and SENP5 preferentially remove monomeric SUMO2/3 moieties, whereas SENP6 and SENP7 selectively act on SUMO2/3 chains and do not participate in the maturation of SUMO proteins (Mikolajczyk et al., 2007). SENP enzymes are themselves controlled by sumoylation, ubiquitylation and subcellular localization (Hickey et al., 2012).

SUMO FUNCTION

The role of sumoylation at the organism level became apparent thanks to studies in budding yeast showing that depletion of Ubc9 causes cell cycle arrest at G2/M (Seufert et al., 1995). Likewise, studies conducted in fission yeast showed that deletion of the Ubc9 homologue *hus5* is not lethal but results in chromosome segregation defects (Al-Khodairy et al., 1995). Data obtained from chicken DT-40 cells showed that Ubc9 is essential for the viability of higher eukaryotic cells and its knockout results in the formation of multiple nuclei, likely due to cytokinesis defects, with a significant proportion of cells entering apoptosis (Hayashi et al., 2002). Studies conducted in mice confirmed the severe phenotype of Ubc9 knockout, with embryonic lethality observed at early post-implantation stage. Furthermore, blastocysts failed to expand after 2 days in culture and displayed defects in chromosome condensation and segregation as well as dysmorphic nuclear envelopes and disruption of nucleoli and PML bodies (Nacerdine et al., 2005). Sumoylation has also been linked to human pathologies, in that human SUMO1 haploinsufficiency was found to be responsible for cleft lip and palate, a finding corroborated by a mouse model (Alkuraya et al., 2006). Others, however, reported no obvious developmental defects in SUMO1 knockout mice (Evdokimov et al., 2008; Zhang et al., 2008), suggesting possible redundancy among SUMO proteins.

THE SUMO ENIGMA

A peculiarity distinguishing SUMO from other PTMs is the ability of triggering fully-fledged responses despite a minor amount of the proteins involved in the response is actually modified by SUMO, a phenomenon denoted as “the SUMO enigma” (Hay, 2005). This occurs in transcriptional repression, where modification by SUMO is apparently required for the recruitment of transcription factors into repressive protein complexes, with their sequestration remaining permanent even upon SUMO removal (Wilkinson and Henley, 2010). SUMO modification of only a small substrate population at any given time point was also suggested to occur for DNA repair proteins such as thymidine-DNA glycosylase (TDG). TDG is part of the base excision repair system (BER) and displays the ability of specifically addressing uracil/thymidine base mismatches (Sancar et al., 2004). The rate-limiting step in the enzymatic reaction carried out by TDG is its dissociation from the abasic site (AP site) generated as first step in the BER process. The high affinity of TDG for the structure generated upon removal of the base is an important self-protection mechanism put in place by the cell since AP sites can turn into DNA strand breaks, thus threatening genome stability (Hardeland et al., 2002). Sumoylation is the appropriate solution to this issue, in that SUMO-modified TDG loses affinity for the abasic site allowing recruitment of the (AP)-endonuclease that acts in the next step of BER (Sancar et al., 2004). To re-initiate the circle, desumoylation by SENPs/ULPs renders TDG promptly available for the next round of lesion recognition and processing (Hardeland et al., 2002). Thus, SUMO modification of minimal amounts of TDG is sufficient to address the repair of uracil/thymidine base mismatches in a highly controlled manner.

PTMs IN DNA DAMAGE RESPONSE: THE OLD AND THE NEW

The cascade of events resulting from detection of DNA damage and orchestrating its repair has been best described for DNA double-strand breaks (DSBs). A detailed account of ubiquitylation and sumoylation events occurring at DSBs will be followed by a brief mention to the signaling triggered by other types of DNA lesions.

DSB RECOGNITION

Initial players consist of proteins or protein complexes such as Ku70 and Ku80 or MRE11/RAD50/NBS1 (MRN) that, through recognition and binding to DNA ends, facilitate recruitment and activation of the protein kinases DNA-PKcs or ATM, respectively. The latter function as transducers of the DNA damage signal and help coordinating repair with checkpoint activation and cell cycle arrest (Sancar et al., 2004). When the sister chromatid is available as template, repair is addressed through the error-free pathway of homologous recombination (HR) rather than the predominant but error-prone pathway of non-homologous end-joining (NHEJ) (Sancar et al., 2004). HR initiates upon recognition of DNA ends by the MRN complex, an event that facilitates recruitment of ATM through direct interaction with the C-terminus of the NBS1 component (Falck et al., 2005) (**Figure 2A**).

ATM is a homodimer and exists in a complex containing the protein phosphatase PP2A, which maintains ATM inactive by catalyzing its constitutive dephosphorylation (Goodarzi et al., 2004), and the histone acetyltransferase Tip60, which is maintained at low level by CUL3-dependent ubiquitylation and plays a role in the modification of chromatin at sites of damage (Murr et al., 2006; Sun et al., 2009). NBS1-dependent ATM recruitment at sites of damage is followed by ATM autophosphorylation at S₁₉₈₁ with ensuing activation of the kinase.

The mechanism of DSB repair operating in the absence of a homologous template for recombination-mediated repair is non-homologous end joining. In this case, DNA ends are bound by the Ku70/Ku80 heterodimer that recruits DNA-PK catalytic subunit, causing inward translocation of the heterodimer and positioning the catalytic subunit at DNA ends. Next, depending on the complexity of the lesion, different processing factors are recruited, such as the endonuclease Artemis and the polynucleotide kinase/phosphatase PNKP. The release of DNA-PKcs from DNA ends, which is induced by autophosphorylation, leads to the final step of the process, with XRCC4, DNA ligase IV and XLF performing ligation of the DNA ends (Dobbs et al., 2010).

SITE MARKING

ATM-mediated phosphorylation of H2AX at the C-terminal S₁₃₉ (γ H2AX) (Rogakou et al., 1998), possibly paralleled by dephosphorylation of Y₁₄₂ (Cook et al., 2009), marks the site of damage and contributes to destabilize nucleosome structure (**Figure 2A**). A critical role in the generation of γ H2AX in response to IR is apparently played by mono-ubiquitylation of the histone at K₁₁₉/K₁₂₀, which facilitates the subsequent recruitment of ATM. H2AX mono-ubiquitylation is catalyzed by a complex composed of the polycomb protein BMI1 and the RING finger proteins RING1 and RNF2 (Ginjala et al., 2011; Pan et al., 2011; Wu et al., 2011). Upon phosphorylation, H2AX acts as docking site

for MDC1 that, by virtue of the high affinity of its C-terminal BRCT tandem repeats for the phospho-S₁₃₉ epitope in γ H2AX, is the first protein localizing at sites of damage (Bekker-Jensen and Mailand, 2010). MDC1 orchestrates the consecutive assembly of factors that will, in turn, mediate the recruitment of DNA repair proteins. Such factors comprise 53BP1, BRCA1, and the E3-Ubiquitin ligase RNF8. Through its N-terminal FHA domain RNF8 binds phosphorylated MDC1 as well as HERC2, with the latter acting as coordinator of Ubiquitin-dependent assembly of DNA repair factors (Bekker-Jensen et al., 2010). SUMO1 modification of HERC2 and RNF168 by the E3-ligase PIAS4 promotes recruitment of RNF8 to the complex and stabilizes the interaction between RNF8 and the E2-conjugating enzyme Ubc13 (Danielsen et al., 2012). In turn, RNF8 contributes to remodel chromatin around sites of damage through a transient K₄₈ and a persistent K₆₃ ubiquitylation of both H2A and H2AX (Huen et al., 2007; Mailand et al., 2007). Histone ubiquitylation was long known as a post-translation modification occurring during transcriptional responses and mono-ubiquitylation of H2A in the context of the cellular response to DNA damage was first described for the repair of UV-induced lesions (Bergink et al., 2006). RNF8 was identified as the E3-ligase catalyzing H2A and H2B mono-ubiquitylation in response to IR (Wu et al., 2009) and UV (Marteijn et al., 2009) and thus proposed to be a conserved element in the initial response to DNA DSBs and UV lesions. H2A ubiquitylation contributes to the recruitment of DNA repair factors. Once bound to DNA, MDC1 is sumoylated at K₁₈₄₀ by PIAS4 in a manner that facilitates its recognition and ubiquitylation by the E3-ligase RNF4, with consequent degradation (Luo et al., 2012). Additional factors recruited to phosphorylated H2AX consist of chromatin remodeling complexes such as INO80 and SWR1 in yeast (Morrison et al., 2004; Van Attikum et al., 2007) and p400 in humans (Xu et al., 2010). K₆₃ histone di-ubiquitylation by RNF8, in turn, allows binding of the adaptor protein RAP80 through its UIM motifs (Sato et al., 2009) and the recruitment of Abraxas (ABRA1), which acts as anchor for BRCA1 at sites of DNA damage (Sobhian et al., 2007; Bekker-Jensen and Mailand, 2010). The BRCA1 complex, in turn, contains the DUB BRCC36, which is able to depolymerize K₆₃ Ubiquitin chains, thus contributing to maintain steady-state levels of Ubiquitin at sites of damage (Shao et al., 2009).

It has been proposed that initial histone ubiquitylation by RNF8 represents a docking signal for RNF168, a second E3-Ubiquitin ligase that is recruited to chromatin to the purpose of amplifying the signal through further ubiquitylation of histones around the site of damage (Doil et al., 2009; Pinato et al., 2009; Stewart et al., 2009). Structural studies on the RING domains of RNF8 and RNF168 supported this view showing that RNF8 can dimerize and as such productively interact with Ubc13/Mms2 and catalyze K₆₃-linked poly-Ubiquitin chains, whereas the monomeric RNF168 does not interact with the E2 enzyme and is by far catalytically less efficient (Campbell et al., 2012). RNF168 features two MIUs (Motif Interacting with Ubiquitin) that are responsible for recognition of di-ubiquitylated K₆₃ on histone H2As and accrual at sites of damage (Doil et al., 2009; Pinato et al., 2009; Stewart et al., 2009). Deletion of the two MIU-domains showed that a small fraction of RNF168 was nonetheless able

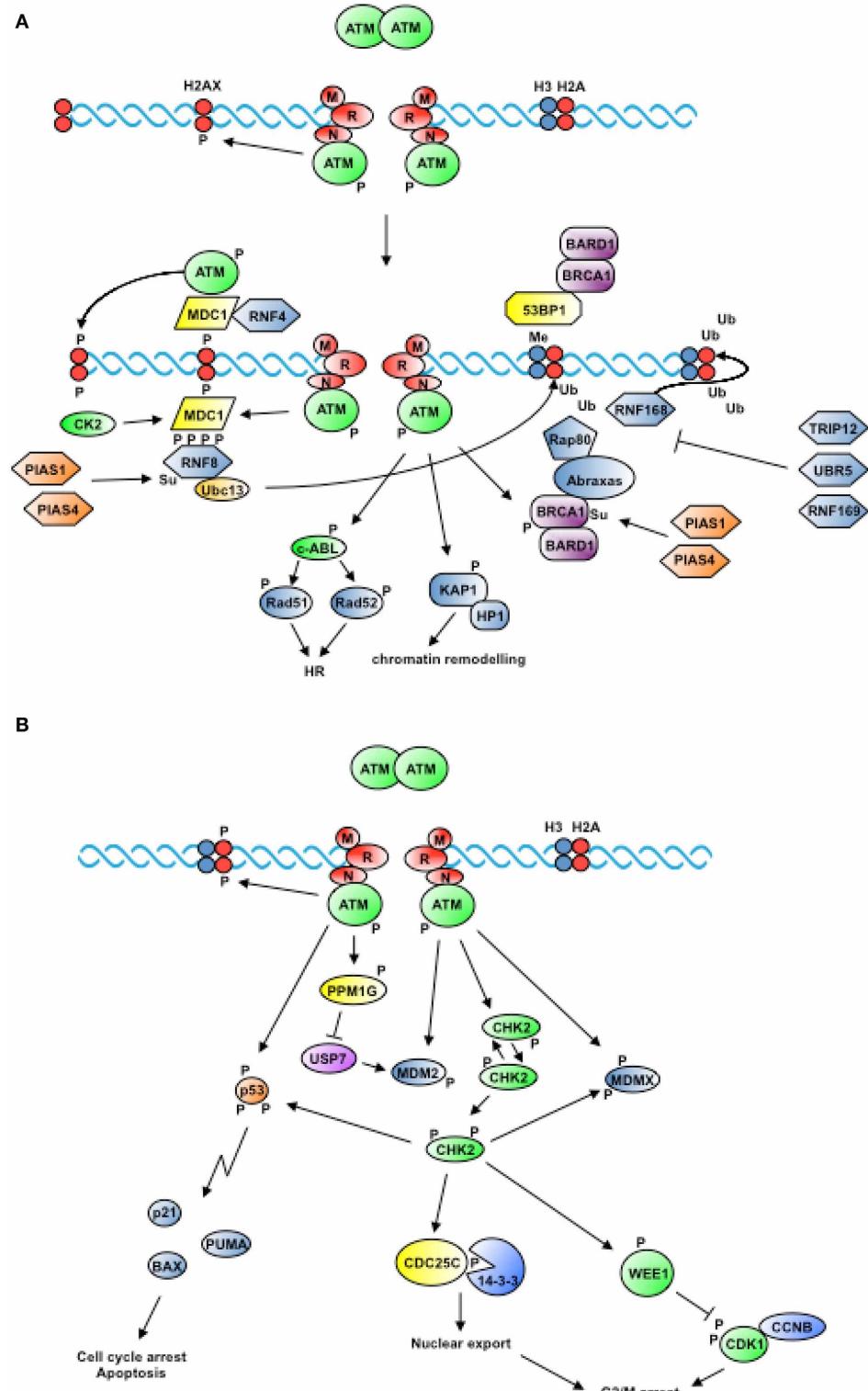


FIGURE 2 | Proximal and widespread DNA damage signals. (A) In response to the generation of DSBs, ATM is recruited to DNA in an MRN-dependent manner and is activated by autophosphorylation. ATM-dependent phosphorylation of H2AX triggers the recruitment of factors that mark the site of damage and cooperate to amplify the signal. In addition, ATM phosphorylates proteins that contribute to

remodel chromatin and promote homologous recombination (see text for details). **(B)** Activation of ATM triggers the phosphorylation of the protein kinase CHK2 among others, which freely diffuses from the site of damage to transduce DNA damage signals to cell cycle regulators, resulting in the inhibition of cell cycle transitions (see text for details).

to bind chromatin (Pinato et al., 2009), leading to the discovery of an additional Ubiquitin-binding domain (UIM- and MIU-related) that is necessary for proper localization of RNF168 at sites of damage (Pinato et al., 2011). A twist to the debate on the hierarchy of E3s recruitment at sites of damage was brought by studies showing that RNF8 is primarily responsible for ubiquitylation of histone H2As at C-terminal sites (K₁₁₈/K₁₁₉), whereas RNF168 catalyzes the mono-ubiquitylation of a set of sites located at the N-terminus of histone H2As (K₁₃/K₁₅) (Gatti et al., 2012; Mattioli et al., 2012). This finding indicated that the order by which E3s are recruited does not predict the order in which they ubiquitylate H2As. The authors suggested that RNF168 might catalyze the priming event at N-terminal sites that, being located on the opposite side of the nucleosome with respect to the RNF8 target sites, may initiate distinct signaling events (Mattioli et al., 2012).

The importance of RNF8-/RNF168-dependent ubiquitylation is well exemplified by the RIDDLE syndrome, where recessive mutations in the *RNF168* gene lead to the expression of aberrant RNF168 protein isoforms, resulting in failure of 53BP1 and BRCA1 accumulation at IR-induced foci and of the subsequent activation of DNA damage responses (Stewart et al., 2009).

Sumoylation of RNF8, RNF168, and BRCA1 mediated by PIAS1 and PIAS4 enhances their E3-ligase activity, contributing to render more efficient histone ubiquitylation at DSBs (Galanty et al., 2009). Through interaction with the Ubiquitin-conjugating UBE2L6/UBCH8, RNF8 controls the degradation of the demethylase JMJD2A/KDM4A resulting in the uncovering of H4K₂₀me2 mark and promoting the recruitment of 53BP1 at DNA damage sites (Mallette et al., 2012). RAD18 is another Ubiquitin E3-ligase recruited at DNA lesions through recognition of K₆₃ ubiquitylated histones and acting downstream of RNF8/RNF168 (Huang et al., 2009).

The boost of DNA damage-induced ubiquitylation events was initially shown to be modulated by deubiquitylating enzymes such as USP3, BRCC36, and OTUB1 (Nicassio et al., 2007; Shao et al., 2009; Nakada et al., 2010). Subsequent studies on mechanisms that control the excessive spreading of histone ubiquitylation around sites of damage demonstrated the involvement of the HECT-domain E3-ligases TRIP12 and UBR5. By determining the amount of RNF168 that is loaded at sites of damage, TRIP12 and UBR5 contribute to optimize the recruitment of physiological amounts of genome caretakers such as 53BP1 and BRCA1 (Gudjonsson et al., 2012). An RNF168 paralog, namely the E3-ligase RNF169, also contributes to limit the spreading of non-proteolytic ubiquitylation at regions flanking DNA damage sites. Specifically, RNF169 MIU2 domain was demonstrated to recognize histones ubiquitylated by RNF8/RNF168, thus outcompeting and limiting the productive recruitment of 53BP1 and RAP80 (Chen et al., 2012; Poulsen et al., 2012). Recognition of Ubiquitin by RNF168 and RNF169 is mediated by modules composed of a UBD juxtaposed to a short targeting sequence, called the LR-motif (LRM), a structure also shared by RAD18 and RAP80 (Panier et al., 2012).

In concomitance with the events described above, phosphorylation of MDC1 by casein kinase 2 (CK2) allows the former to capture additional molecules of ATM that phosphorylate both

H2AX in the neighboring nucleosomes and MDC1 itself (Polo and Jackson, 2011). Phosphorylation does not only serve the function of promoting the assembly of DNA repair modules, but also contributes to break up interactions to facilitate repair processes. This is the case of the transcriptional repressor and RING finger protein KAP1/TIF1β/TRIM28, which is released from chromatin upon ATM-mediated phosphorylation of S₈₂₄, an event that results in the dissociation of heterochromatin protein 1 (HP1) from chromatin and contributes to remodel regions that will undergo repair (Goodarzi et al., 2008). Chromatin relaxation in response to DSBs apparently consists of two stages: an early step that occurs before the generation of γH2AX and that is ATP-dependent (Kruhlak et al., 2006) and a second step that relies on the recruitment of a fraction of the RNF20/RNF40 heterodimer to sites of damage where it catalyzes the mono-ubiquitylation of H2B (Moyal et al., 2011). Mechanistically, it was demonstrated that mono-ubiquitylation of H2B is sufficient to interfere with the compaction of chromatin (Fierz et al., 2011).

DNA END RESECTION

Marking DNA double-strand break sites is followed by the recruitment of repair proteins in charge of processing DNA ends to create structures that are suitable to recombination. This task is initially accomplished by the MRN complex that in conjunction with CtIP/RBBP8 carries out initial trimming at the break, a step that is followed by extensive processing of DNA ends by the redundant function of EXO1 and the DNA2/BLM complex (Mimitou and Symington, 2009; Eid et al., 2010). Proteins participating in DNA processing are also controlled by PTMs.

CtIP is phosphorylated in a CDK-dependent manner in S and G2 phases of the cell cycle at T₈₄₇ and S₃₂₇. Whereas phosphorylation of the former affects resection activity, modification of the latter influences BRCA1 binding (Yu and Chen, 2004; Huertas and Jackson, 2009). In response to DSBs CtIP is additionally phosphorylated by ATM (Matsuoka et al., 2007). Binding of BRCA1/BARD1 to CtIP is mediated by the BRCT domain of BRCA1 and causes ubiquitylation of CtIP in a manner that does not target it to degradation but facilitates binding to DNA and enrichment at sites of damage (Yu et al., 2006). This is an example of ubiquitylation as means to selectively target protein to a defined region in the cell or to a structure.

In response to stalled replication, EXO1 protein level is controlled by ATR-dependent phosphorylation and poly-ubiquitylation catalyzed by a currently unknown E3 ligase (El-Shemery et al., 2005, 2008). On the other hand, both in yeast and man EXO1 nuclease activity is controlled by PIKK-dependent phosphorylation upon induction of DSBs (Morin et al., 2008; Bolderson et al., 2010). Sumoylation of EXO1 has also been reported (Tatham et al., 2011), though its functional significance awaits clarification.

The Bloom syndrome helicase (BLM) plays an important role in homologous recombination and in the repair of damaged replication forks (Jones and Petermann, 2012). Modification of BLM by SUMO is necessary for a balanced γH2AX response in HU treated cells, with cells that express SUMO-deficient forms of BLM displaying excessive γH2AX phosphorylation, accumulation of DNA breaks and hypersensitivity to DNA damage (Ouyang

et al., 2009). In HU-treated cells expressing SUMO-deficient forms of BLM, the ability to localize RAD51 at damaged replication forks is compromised and sister-chromatid exchanges does not occur. This led to the suggestion that sumoylation represents a switch between pro- and anti-recombinogenic roles for BLM in HR (Ouyang et al., 2009).

Resection of DNA ends by EXO1 or the BLM/DNA2 complex leads to the formation of long 3'-overhangs that are the structures participating in homologous recombination. Replication Protein A (RPA) is the major ssDNA binding protein complex present in eukaryotes and consists of three subunits: RPA1 (70 kDa), RPA2 (32 kDa), and RPA3 (14 kDa). RPA1 has high affinity for DNA and is the docking subunit for a number of proteins involved in DNA synthesis and repair (Fanning et al., 2006). RPA2 has lower affinity for DNA and, thanks to its C-terminal winged helix domain, binds weakly but specifically to AID, to BER proteins such as UDG or to NER proteins such as XPA (Fanning et al., 2006). RPA2 is the major target of phosphorylation events that occur during DNA replication and the DNA damage response. RPA3 is the only component with no affinity for DNA but playing an important role in the stabilization of the trimeric protein complex (Fanning et al., 2006). It has been observed that association between the SUMO protease SENP6 and RPA1 during transition through S-phase maintains RPA1 in a hypo-sumoylated state. Camptothecin-induced DSBs weaken the interaction between RPA1 and SENP6, facilitating RPA1 sumoylation at K₄₄₉ and K₅₇₇, an event that results in increased interaction with Rad51 and displacement of RPA from the ssDNA filament (Dou et al., 2010).

Sumoylation of MRE11 and RAD54 has also been reported (Tatham et al., 2011), though the functional significance of this PTM is as yet unknown.

PROXIMAL AND DISTAL SIGNALING

In addition to the two members of the PIKK family of protein kinases mentioned above in the context of DSB recognition, namely ATM and DNA-PK, also ATR participates in orchestrating the overall response to genotoxic damage. An important component of the DNA damage response is the transduction of signals to the cell cycle machinery. Unlike ATM or DNA-PK that are activated by DNA ends (Uematsu et al., 2007; You et al., 2007), ATR activation specifically depends on the presence of ssDNA resulting from the processing of different types of damage (Zou and Elledge, 2003) or naturally occurring at replication forks (MacDougall et al., 2007). ATR triggering typically occurs after ATM activation (Jazayeri et al., 2006). Two checkpoint kinases are phosphorylated by ATR at S₃₁₇ and S₃₄₅ (CHK1) (Zhao and Piwnica-Worms, 2001) and by ATM or ATR at seven residues in the N-terminal domain (CHK2) (Matsuoka et al., 2000), respectively. This triggers homo-dimerization of the checkpoint kinases and full activation through auto-phosphorylation (Lee and Chung, 2001) (**Figure 2B**).

The ATR-CHK1 pathway controls the timing of DNA replication origin firing during regular transition through S-phase (Shechter et al., 2004) and triggers G2/M arrest in response to γ -irradiation (Liu et al., 2000). ATR-mediated phosphorylation of CHK1 at S₃₄₅ exposes a degron-like region at the C-terminus of the kinase allowing recognition by cytoplasmic Cul1/FBX6

or nuclear Cul4A/CDT2 SCF E3-ligase complexes that promote poly-ubiquitylation and degradation of CHK1 (Zhang et al., 2009; Huh and Piwnica-Worms, 2013). It has been proposed that proteolysis of activated CHK1 results in checkpoint termination (Zhang et al., 2005b).

The ATM/CHK2 axis controls both transient and sustained cell cycle arrest following detection of DNA damage (**Figure 2B**) (Shiloh and Ziv, 2013). Namely, by phosphorylating CDC25 phosphatases and the WEE1 kinase, CHK2 blocks cell cycle transitions mediated by Cyclin-CDKs, whereas by phosphorylating p53, MDM2, and PML it promotes apoptosis (Antoni et al., 2007).

Both CHK1 and CHK2 impinge on the machinery driving cell cycle transitions by directly phosphorylating controllers of cyclin-dependent kinases such as the WEE1 kinase and the CDC25A and CDC25C phosphatases (Bartek et al., 2004) (**Figure 2B**). WEE1 catalyzes phosphorylation of two residues in the Gly-rich P-loop of CDK1, namely T₁₄, and Y₁₅, in a manner that does not affect nucleotide binding but hampers catalysis (Ferrari, 2006). CDC25 phosphatases specifically remove the phosphate from the two residues in the ATP-binding site of CDKs, causing full activation of Cyclin/CDK complexes (Ferrari, 2006). Inhibition of CDC25C by DNA damage essentially occurs by a 14-3-3-mediated sequestration mechanism, whereas CDC25A degradation via Ubiquitin-proteasome pathways is a primary control mechanism both in dividing cells and in response to DNA damage (Donzelli and Draetta, 2003). Phosphorylation of CDC25A on S₇₆ by CHK1 (Jin et al., 2008a) serves as priming event to facilitate phosphorylation on S₇₉ and S₈₂ by protein kinase CK1 or glycogen synthase kinase 3 β (GSK-3 β) (Kang et al., 2008; Honaker and Piwnica-Worms, 2010). This, in turn, allows recruitment of the SCF β -TrcP E3 ligase that promotes CDC25A poly-ubiquitylation (Busino et al., 2003).

DNA DAMAGE RECOVERY

Following completion of DNA repair, cell cycle restart is contributed by degradation of molecules that were involved both in signaling DNA damage and in blocking cell cycle progression. This is the case of the adaptor protein Claspin, which is targeted by SCF β -TrcP upon PLK1-dependent phosphorylation (Mameli et al., 2006; Peschiaroli et al., 2006) and whose level is maintained low throughout G1 by the APC/CDH1 E3-ligase (Bassermann et al., 2008), and of the kinase WEE1 (Bartek and Lukas, 2007).

OTHER DNA LESIONS

Additional examples of regulation of DNA damage responses by ubiquitylation are provided by Fanconi Anemia (FA), Translesion DNA Synthesis (TLS) and Nucleotide Excision Repair (NER). FA is an X-linked disease characterized by mutations in genes coding for factors of this DNA repair pathway. Upon exposure to DNA interstrand cross-linking (ICL) agents, FANC proteins form a nuclear “core-complex” in which FANCL is the E3 Ubiquitin ligase responsible, together with its cognate E2 UBE2T, for the mono-ubiquitylation of FANCI and FANCD2 (the ID-complex) on residues K₅₆₁ and K₅₂₃, respectively. This is an event required for the formation of damage-induced foci (Wang, 2007). Mutation of the Ubiquitin-binding domain on FANCI-FANCD2 results in hypersensitivity to mitomycin C or cisplatin

(Smogorzewska et al., 2007). In TLS, which represents one of the main mechanisms allowing DNA lesion bypass in S-phase (Waters et al., 2009), ubiquitylation of PCNA plays a key role (see below). BER addresses the repair of modified bases or abasic sites resulting from depurination/depyrimidination events (Almeida and Sobol, 2007). In addition to TDG, which participates in lesion recognition and processing, the BER scaffold component XRCC1 is controlled by phosphorylation (Loizou et al., 2004) and sumoylation (Gocke et al., 2005). The E3-ligase CHIP/STUB1 adds another layer of control to BER by mediating ubiquitylation of the pool of XRCC1 and Pol β that are not directly participating in the process of lesion recognition and repair (Parsons et al., 2008).

INTERDEPENDENCE OF PTMs

HIERARCHICAL PRIMING

An interesting feature of PTMs is their reciprocal influence, as clearly established for histones, where the antagonism or the synergism of certain modifications defines a “code” that guides protein-DNA interactions (Sims and Reinberg, 2008). These, in turn, influence the compaction of chromatin and ultimately affect biological responses such as transcription, DNA replication and DNA repair (Kouzarides, 2007). Such effects can be cumulative or exclusive, with a clearly defined hierarchy of PTMs affecting a given target protein. In the DNA damage response a notable example of consecutive PTMs occurring in a hierarchical manner is represented by FEN1, the flap endonuclease responsible for cleavage of single stranded 5' overhangs in Okazaki fragments during DNA replication and also involved in DNA repair. Phosphorylation at S₁₈₇ in FEN1 catalytic domain by cyclin A/CDK2 results in its release from PCNA, the DNA polymerase processivity factor that stimulates FEN1 nuclease activity (Henneke et al., 2003). Subsequent modification of K₁₆₈ in FEN1 by SUMO3 facilitates K₃₅₄ ubiquitylation by the E3 ligase PRP19, resulting in FEN1 degradation at the end of S-phase, an event that contributes to ensure a timely transition to G2 (Guo et al., 2012).

COMPETITION FOR THE SUBSTRATE

In addition to the ability of sumoylation to directly alter the properties of the protein undergoing this modification, it may also serve as a competitor to other PTMs. Indeed, since sumoylation targets lysine residues in the substrate, similarly to ubiquitylation, methylation or acetylation, the modification of one or more lysine in the substrate could block other PTM machineries from accessing these residues, thus indirectly affecting protein function (Walsh et al., 2005). Established examples of competition among PTMs are RanGAP1, which upon sumoylation preferentially binds the nuclear pore complex (Melchior, 2000), the NF- κ B signaling pathway (Huang et al., 2003) and PCNA (Hoege et al., 2002). Specifically to the latter, early studies showed that mono-ubiquitylation mediated by RAD6 (E2) and RAD18 (E3), K₆₃ poly-ubiquitylation by MMS2, UBC13, and RAD5 and sumoylation by UBC9 all affect the same lysine residue (K₁₆₄) (Hoege et al., 2002). Subsequent work clearly established that PCNA mono-ubiquitylation supports translesion synthesis, a pathway allowing stalled DNA replication to proceed beyond damage through the replacement of processive polymerases with

specialized polymerases (Bienko et al., 2005; Garg and Burgers, 2005). K₆₃ poly-ubiquitylation, on the other hand, facilitates synthesis by a template-switch mechanism, a complex but essentially error-free pathway that utilizes the undamaged, newly synthesized daughter strand of the sister chromosome as template (Branzei and Foiani, 2010). Finally, PCNA sumoylation prevents the formation of DSBs and the occurrence of inappropriate recombination events at stalled DNA replication forks by a mechanism involving the anti-recombinogenic activity of the helicase Srs2 in yeast (Papouli et al., 2005; Pfander et al., 2005) and possibly by a similar mechanism in humans (Gali et al., 2012).

CROSS-TALKING

A number of Ubiquitin E3-ligases display the ability to bind SUMO chains on proteins that, in turn, become their substrates. A reported case is PML, which undergoes modification by SUMO-1 as well as by SUMO-2/3. Whereas attachment of SUMO-1 determines confinement of the protein in PML nuclear bodies (Muller et al., 1998) formation of SUMO2/3 chains facilitates the recruitment of the E3-ligase RNF4, which ubiquitylates the SUMO chains and ultimately targets PML to degradation (Lallemand-Breitenbach et al., 2008; Tatham et al., 2008; Weisshaar et al., 2008). RNF4 displays the ability to interact with other sumoylated substrates, such as MDC1 and RPA, via its N-terminal SUMO interaction motif (SIM) and to subsequently regulate their stability (Galanty et al., 2012). Another interesting case is BRCA1, which co-localizes with and is sumoylated by PIAS1 and PIAS4 at sites of damage. This, in turn, was reported to enhance BRCA1 E3-ligase activity possibly through a SUMO-dependent increase of the E3-E2 interface (Morris et al., 2009).

SYNERGY

The advent of proteome-wide studies allowed appreciating the fact that, like phosphorylation, sumoylation triggered by a defined stimulus or stress does not target a single components but a vast majority of the protein machinery involved in the response. Work conducted in yeast established that lack of overall sumoylation in a hypomorphic mutant of the SUMO E2 Ubc9 impaired survival in response to DNA damage (Cremona et al., 2012). This resulted from incomplete replication of damaged DNA as well as defective resection at DSBs. The authors found that DNA damage-induced sumoylation occurred independently of phosphorylation events that were triggered by the checkpoint and was proposed to act in parallel with them to support cell survival (Cremona et al., 2012). A study conducted using SILAC-based mass spectrometry identified 844 different SUMO conjugates, the abundance of which did not seem to change in response to DNA damage (Psakhye and Jentsch, 2012). Interestingly though, the set of sumoylated proteins enriched in response to DNA damage was specifically that of the HR machinery. The authors found that DNA end resection and the consecutive generation of long ssDNA tracts acted as trigger to the wave of sumoylation that characterized the response. Sumoylation of HR proteins was found to occur independently and in parallel, with no influence of one sumoylation event on the other, and to entirely depend on the SUMO E3-ligase Siz2. Sumoylation promoted physical

interaction among HR proteins, thus facilitating DNA repair (Psakhye and Jentsch, 2012).

UBIQUITIN AND SUMO AS TARGETS IN CANCER THERAPY

Ubiquitylation and/or sumoylation defects have been implicated in the pathogenesis of a number of human diseases among which is cancer (Sun, 2006; Bettermann et al., 2012).

UBIQUITIN AND CANCER

Examples of over-expression of ubiquitylation pathway components in cancer cells are the p53-specific ARF-BP1/Mule HECT E3-ligase, the F-box proteins SKP2 and β -TrcP1, the SCF component Cul-4A and the RING-finger proteins RNF11, ZNF164 (Chen et al., 2006), and RNF5 (Bromberg et al., 2007). Mutation or deletion of E3-ligases that normally function as tumor suppressors has also been reported. This is the case of the RING-finger E3-ligases BRCA1/BARD1 and SIAH1 (Chen et al., 2006). Finally, epigenetic inactivation of genes coding for the E3-ligase HACE1 (Hibi et al., 2008) or the RING-finger protein CHFR (Chen et al., 2006) has been observed in several types of carcinomas.

Based on the reasoning that E3 enzymes are druggable targets, pharmaceutical companies embarked on high-throughput screenings in search for compounds that would target the active site of E3-ligases or block interaction with their substrates (Sun, 2006; Hoeller and Dikic, 2009). A notable example of the latter is Nutlin, which impairs the p53-HDM2 interaction by filling a groove in HDM2 where p53 is accommodated (Vassilev, 2007). Despite the initial enthusiasm raised by Nutlin and its derivatives, the limitation of its efficacy in cells expressing wild-type p53 excluded their use from a number of other cancers. More discouraging, the cytostatic effect of Nutlins in p53-deficient cells indicated that they did not solely inhibit the p53/HDM2 interaction (Vanderborght et al., 2006). The p53-targeting molecule RITA (NSC652287), identified in a screening conducted on a pair of isogenic cell lines differing only in their p53 status, was shown to bind p53 N-terminus (Issaeva et al., 2004). However, RITA did not specifically target the p53-HDM2 dimer but also other p53 protein complexes (Hjerpe and Rodriguez, 2008). Similar issues were encountered with other inhibitors of E3-ligases (Guedat and Colland, 2007).

Another interesting case of targeting E3-ligases is BRCA1. Synthetic lethality was observed when PARP-inhibitors are administered to cells of BRCA-deficient patients (Bryant et al., 2005; Farmer et al., 2005). Considering that BRCA deficiency occurs in <5% of breast cancers, the results obtained with PARP-inhibitors prompted studies attempting to exploit the concept of synthetic lethality in non-mutation carriers. Specifically, small molecules targeting the phospho-dependent interaction of BRCA1 with partners such as Abraxas were administered to breast and cervical cancer cells to mimic the inactivating mutation of the otherwise wild-type BRCA1 gene. The data showed that, under these conditions, PARP-inhibitors effectively sensitized cells to IR-induced damage (Pessetto et al., 2012).

The E1-activating enzyme and the proteasome have also been considered as possible targets, with the *caveat* that inhibiting the ubiquitylation cascade at its apex may impair pathways of vital importance to the survival of normal cells. This is

particularly true if one considers the widespread use of ubiquitylation in the control of cellular functions. Nonetheless, inhibitors of the chymotryptic activity of the proteasome have been identified and characterized. Compounds such as bortezomib have received approval from FDA and are currently used for the treatment of multiple myeloma and mantle cell lymphoma (Guedat and Colland, 2007; Rastogi and Mishra, 2012). Similarly, ATP-competitive inhibitors blocking the transfer of Ubiquitin from the E1-activating enzyme to E2-conjugating components of the cascade have been identified (Guedat and Colland, 2007).

Inhibition of deubiquitylating enzymes has also been explored as possible alternative to the development of inhibitors of the ubiquitylation cascade. A compound specifically targeting USP7 was shown to stabilize p53, activate p53-dependent transcription, block cell growth and induce apoptosis (Guedat and Colland, 2007). Recently, a novel strategy based on the use of combinatorial libraries of Ubiquitin variants has led to the identification of mechanisms of DUBs inhibition and provided the demonstration that this approach could be applied to the discovery of specific E2 or E3 inhibitors (Ernst et al., 2013).

SUMO AND CANCER

With regard to the role of SUMO in cancer, Ubc9/UBE2I was found overexpressed in ovarian carcinoma specimens (Mo et al., 2005). Xenografts studies conducted in mice revealed that tumors expressing wildtype Ubc9 grew better than controls, while tumors expressing dominant negative Ubc9 exhibited reduced growth (Mo et al., 2005). A comprehensive study reported an increase in UBC9 expression in primary colon and prostate cancer compared with their normal tissue counterparts, whereas UBC9 levels were found lower in metastatic breast, prostate, and lung cancer in comparison with their corresponding normal and primary adenocarcinoma tissues (Moschos et al., 2010). Increased UBC9 expression was also observed in melanoma-infiltrated lymph nodes, with depletion of UBC9 resulting in sensitization of melanomas to the cytotoxic effects of topotecan and cisplatin (Moschos et al., 2007). A comprehensive collection of studies on UBC9 mRNA expression pattern in different cancer types can be found at www.nextbio.com. Based on these findings, targeting UBC9 in cancer therapy was initially proposed (Mo et al., 2005). However, given the widespread use of sumoylation as PTM controlling numerous metabolic pathways, altering the overall pattern of sumoylation in the cell was countered by others as a non-specific and ineffective method to combat cancer (Bawa-Khalfe and Yeh, 2010). Support to arguments in favor of UBC9 as valid target in cancer therapy is provided by its pattern of differential expression, with higher levels of UBC9 in cancerous vs. normal tissues, offering a possible therapeutic window (Mo and Moschos, 2005). In this respect, crystallographic studies mapping the surfaces in UBC9 involved in the interaction with specific E3s and their substrates represent a promising avenue to the design of small compounds disrupting selective sumoylation reactions (Mo and Moschos, 2005).

Increased levels of the desumoylating enzyme SENP1 were reported in thyroid oncocytic adenocarcinoma (Jacques et al., 2005) and prostate cancer (Cheng et al., 2006). A transgenic mice model showed that overexpression of Senp1 in the prostate led

to the development of prostatic intraepithelial neoplasia at an early age (Cheng et al., 2006). Promising results have been obtained in studies aiming at the identification of SUMO-specific protease (SENP) inhibitors (Hemelaar et al., 2004; Borodovsky et al., 2005) or based on the screening of cysteine-protease inhibitor libraries (Albrow et al., 2011). The latter, in particular, led to the identification of two classes of compounds: the first, containing a reactive aza-epoxide electrophile linked to an extended peptide backbone and the second, containing an acyloxymethyl ketone reactive group. Structure-activity relationship studies led to the design of covalent inhibitors of multiple hSENPs displaying micromolar IC₅₀ values (Albrow et al., 2011).

Arsenic trioxide, which induces differentiation of leukemic blasts and clinical remission, was shown to promote SUMO-dependent poly-ubiquitylation of PML-RAR α by the Ubiquitin E3-ligase RNF4, with consequent degradation of the fusion protein responsible for acute promyelocytic leukemia

(Lallemand-Breitenbach et al., 2008; Tatham et al., 2008). Thus, in addition to classic approaches based on the chemical inhibition of enzymatic activity, the case of arsenic trioxide illustrated that among the variety of possible avenues to inhibit function, the exploitation of existing pathways in the cell that may be triggered at will is an important option.

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BLM SUMOylation regulates ssDNA accumulation at stalled replication forks

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Polymerase stalling results in uncoupling of DNA polymerase and the replicative helicase, which generates single-stranded DNA (ssDNA). After stalling, RAD51 accumulates at stalled replication forks to stabilize the fork and to repair by homologous recombination (HR) double-strand breaks (DSBs) that accumulate there. We showed recently that SUMO modification of the BLM helicase is required in order for RAD51 to accumulate at stalled forks. In order to investigate how BLM SUMOylation controls RAD51 accumulation, we characterized the function of HR proteins and ssDNA-binding protein RPA in cells that stably expressed either normal BLM (BLM+) or SUMO-mutant BLM (SM-BLM). In HU-treated SM-BLM cells, mediators BRCA2 and RAD52, which normally substitute RAD51 for RPA on ssDNA, failed to accumulate normally at stalled forks; instead, excess RPA accumulated. SM-BLM cells also exhibited higher levels of HU-induced chromatin-bound RPA than BLM+ cells did. The excess RPA did not result from excessive intrinsic BLM helicase activity, because *in vitro* SUMOylated BLM unwound similar amounts of replication-fork substrate as unSUMOylated BLM. Nor did BLM SUMOylation inhibit binding of RPA to BLM *in vitro*; however, in immunoprecipitation experiments, more BLM-RPA complex formed in HU-treated SM-BLM cells, indicating that BLM SUMOylation controls the amount of BLM-RPA complex normally formed at stalled forks. Together, these results showed that BLM SUMOylation regulates the amount of ssDNA that accumulates during polymerase stalling. We conclude that BLM SUMOylation functions as a licensing mechanism that permits and regulates HR at damaged replication forks.

Keywords: Bloom's syndrome, DNA repair foci, homologous recombination, RecQ DNA helicases, replication fork stability

INTRODUCTION

DNA replication is a fundamental process in all living organisms in which the genetic material is duplicated. Highly regulated checkpoint and DNA repair mechanisms ensure that the genome is faithfully replicated with each round of cell division. In mammalian cells, homologous recombination (HR) is an essential repair mechanism that stabilizes damaged DNA replication forks, repairs double-strand breaks (DSBs) that occur during DNA replication, and helps restore productive DNA synthesis following disruption or breakage of replication forks. Although HR is critical in maintaining genome integrity during replication, it is tightly regulated to avoid harmful outcomes.

In the autosomal recessive, clinical entity Bloom's syndrome (BS), genome integrity is strikingly destabilized due to null mutations in the gene *BLM* (Ellis et al., 1995). The BLM protein is an ATP-dependent DNA helicase of the RecQ family, and it possesses multiple functions in DNA replication and HR (Lambert et al., 2010; Wechsler et al., 2011). BLM is one of the first components to be recruited to sites of DNA replication after treatment with agents that inhibit fork progression where it is thought to stabilize stalled forks (Davalos and Campisi, 2003; Sengupta et al., 2003, 2004). In HR-mediated DSB repair, BLM together

with exonucleases EXO1 and DNA2 promote resection of DSBs, generating 3' single-stranded DNA (ssDNA) tails that provide a substrate for loading of the RAD51 recombinase (Nimonkar et al., 2008, 2011; Mimitou and Symington, 2009). BLM preferentially unwinds substrates that resemble recombination intermediates, such as X-junctions and D-loops, and it is a member of a complex, which includes TopIIIα, BLAP75, and BLAP18 that possesses the unique capacity to dissolve a late recombination intermediate, the double Holliday junction, such that only non-crossover products are generated (Wu and Hickson, 2003; Raynard et al., 2006; Wu et al., 2006; Bussen et al., 2007; Singh et al., 2008; Xu et al., 2008). This activity is also important at sites of termination of DNA replication, because BLM accumulates on late replication intermediates to assist in duplex separation so that DNA replication can be efficiently completed (Chan et al., 2007, 2009; Lukas et al., 2011; Barefield and Karlseder, 2012).

BS cells, which lack BLM activity, display numerous characteristics that are the consequence of excessive HR, including high rates of loss of heterozygosity (Langlois et al., 1989; Groden et al., 1990; LaRocque et al., 2011), excessive chromosome abnormalities, such as telomere fusions, ring chromosomes, and quadriradial chromosomes (German, 1964; German and Crippa, 1966), and a high rate of sister chromatid exchange

(SCE) (Chaganti et al., 1974). In addition, BS cells exhibit defects in DNA replication that might lead to excess HR, including accumulation of abnormal DNA replication intermediates (Lönn et al., 1990; Li et al., 2004), slower than normal DNA-chain growth (Hand and German, 1975; Rao et al., 2007), and abnormal origin firing (Davies et al., 2007). BS cells are hypersensitive to replication inhibitors such as hydroxyurea (HU) (Davies et al., 2004), and replication forks in BS cells recover inefficiently from HU-induced stalling, exhibiting accelerated accumulation of DSBs after release (Davies et al., 2007; Ouyang et al., 2009; Sidorova et al., 2013).

In our previous work, we showed that BLM undergoes post-translational modification at lysines K317 and K331 by SUMO-1 and SUMO-2 (Eladad et al., 2005); cells that expressed a GFP-BLM that was mutated at these two SUMO-acceptor sites, which we refer to as SUMO-mutant BLM (SM-BLM) cells, exhibited impairments of replication-associated HR, as evidenced by increased HU-induced DNA damage and reduced HU-induced SCE (Ouyang et al., 2009). We further showed that SM-BLM cells have a defect in the recruitment or retention of the RAD51 recombinase at stalled replication forks and that SUMOylation of BLM *in vitro* increased the binding efficiency between BLM and RAD51, suggesting that BLM SUMOylation could act as a switch to turn on BLM's function in HR repair of stalled forks (Ouyang et al., 2009). Although our data indicates that SUMOylation of BLM regulates the recruitment or retention of RAD51 at stalled replication forks, the mechanism underlying the defect in RAD51 localization in SM-BLM cells remains unclear.

Replication inhibitors such as HU cause stalling of replicative polymerases on DNA, uncoupling of the replicative helicase from polymerases, and the generation of excess ssDNA (Byun et al., 2005). After HU treatment, excess ssDNA is detectable within minutes as evidenced by the accumulation of focal concentrations of ssDNA binding protein—replication protein A (RPA)—at stalled replication forks (Balajee and Geard, 2004; Petermann et al., 2010). In the HR pathway, RAD51 is normally loaded onto RPA-bound ssDNA by a process that involves mediators (e.g., BRCA2 and RAD52) that substitute RAD51 for RPA on ssDNA (Heyer et al., 2010); however, after treatment of normal cells with

HU, RAD51 does not accumulate at stalled replication forks for several hours (Saintigny et al., 2001; Petermann et al., 2010). We hypothesize that a licensing mechanism exists that controls the loading of RAD51 onto ssDNA at stalled replication forks. The licensing mechanism could prevent premature loading of RAD51 at stalled replication forks and activate HR after fork breakage. To explain the deficit of RAD51 loading at stalled forks in SM-BLM cells, we hypothesized that SUMOylation of BLM is a key step in the licensing mechanism. To test this hypothesis, we analyzed the function of mediators and RPA at stalled forks in HU-treated SM-BLM cells.

RESULTS

MEDIATOR ACCUMULATION AT STALLED REPLICATION FORKS IS IMPAIRED IN SUMO-MUTANT BLM CELLS

Because the loading mechanism of RAD51 onto RPA-bound ssDNA requires recruitment of mediators to the repair site (Thorslund et al., 2010), we tested whether the mediators BRCA2 and RAD52 localized normally in HU-treated SM-BLM cells. As expected, treatment of BLM+ cells with 0.5 mM HU for 24 h induced an increase in BRCA2 foci from 10.4 foci/cell to 22.9 foci/cell. On the contrary, despite the presence of approximately twice the number of γ -H2AX and BLM foci, untreated SM-BLM cells exhibited 14.4 BRCA2 foci/cell and HU treatment failed to induce a significant increase in BRCA2 foci to 16.4 foci/cell (Figure 1A). Similarly, whereas HU treatment of BLM+ cells induced an increase in RAD52 foci from 22.6 foci/cell to 35.1 foci/cell, HU treatment of SM-BLM cells resulted in virtually no change in RAD52 foci (from 26.4 foci/cell in untreated cells to 24.9 foci/cell in HU-treated cells; Figure 1B). These data suggested that the impairment of RAD51 localization in SM-BLM cells was explained by an upstream defect in mediator accumulation at stalled forks.

EXCESS RPA ACCUMULATES AT STALLED REPLICATION FORKS IN SUMO-MUTANT BLM CELLS

Because accumulation of BRCA2 and RAD52 mediators at stalled forks was impaired after HU treatment, we reasoned that the impairment could be caused by less ssDNA accumulation after

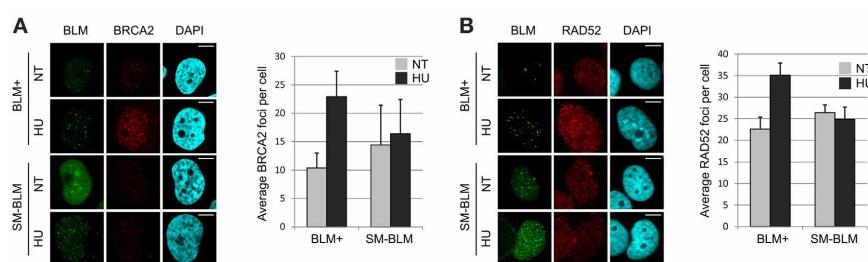


FIGURE 1 | Mediator accumulation at stalled replication forks is impaired in SUMO-mutant BLM cells. (A) Immunofluorescence images of BLM+ and SM-BLM cells untreated (NT) or treated with 0.5 mM HU for 24 h (HU) and stained for BRCA2, with graphical representation of mean numbers of BRCA2 foci. **(B)** Same as **(A)** but stained for RAD52, with graphical representation as in **(A)**. Two independent experiments were carried out on each of two BLM+ and

two SM-BLM clones. Error bars represent the standard deviations of the results of each experiment. After treatment with HU, there were significantly more BRCA2 and RAD52 foci in BLM+ cells ($p < 0.001$ for both comparisons) but not in SM-BLM cells ($p = 0.64$ for BRCA2 and $p = 0.38$ for RAD52). P -values were calculated using mixed effects linear models as described in Materials and Methods. Bars indicate 10 μ m.

HU treatment. To test this possibility, we compared the accumulation of RPA at stalled forks in SM-BLM and BLM+ cells. We treated cells with or without 0.5 mM HU for 24 h, then quantified RPA foci by scoring the number of RPA foci/cell (**Figure 2A**). Untreated SM-BLM cells exhibited a higher number of RPA foci than untreated BLM+ cells (21.1 foci/cell vs. 3.7 foci/cell, respectively). HU induced RPA foci in both SM-BLM and BLM+ cells (105.8 foci/cell vs. 40.5 foci/cell, respectively; **Figure 2B**). The absolute increase was 84.7 foci/cell in SM-BLM cells compared to 36.8 foci/cell in BLM+ cells. We noted that in HU-treated conditions in both SM-BLM and BLM+ cells, BLM co-localized to a high degree with a subset of RPA foci. These results ruled out the possibility that BLM SUMOylation is required for ssDNA accumulation; on the contrary, SM-BLM cells contained a vast excess of RPA foci in both untreated and HU-treated conditions. These data indicated that more ssDNA accumulated at stalled replication forks in SM-BLM compared to BLM+ cells.

EXCESS CHROMATIN-BOUND RPA IN SM-BLM CELLS

Because SM-BLM cells accumulated excess RPA foci, we predicted that more RPA would be bound to chromatin. To test this prediction, we isolated chromatin and nucleoplasmic fractions from BLM+ and SM-BLM cells untreated or treated with 5 mM HU for 6 h and analyzed extracted proteins by immunoblot. In untreated conditions, the ratio of chromatin-bound RPA to total RPA was approximately equal in BLM+ and SM-BLM cells. After HU treatment, however, RPA shifted from nucleoplasmic fractions to chromatin-bound fractions in response to replication stress (**Figure 3A**). The ratio of chromatin-bound RPA to total RPA increased four fold in SM-BLM cells compared to two fold in BLM+ cells (**Figure 3B**). The increased amount of chromatin-bound RPA after HU treatment in SM-BLM cells was consistent

with the indirect immunofluorescence data that showed the accumulation of excess RPA foci. Together, these data indicated that BLM SUMOylation functions to limit the formation of excess RPA-ssDNA complex at stalled replication forks.

DNA HELICASE ACTIVITY OF SUMOYLATED BLM IS NORMAL

The excessive accumulation of fork-associated RPA raised the possibility that excess ssDNA was generated at stalled forks in SM-BLM cells due to a failure to suppress BLM's DNA unwinding activity at the fork. To test this hypothesis, we compared the DNA helicase activity of SUMOylated and unSUMOylated BLM *in vitro*. Purified His-tagged BLM helicase was SUMOylated *in vitro* using purified human E1, UBC9, and SUMO-2 in a reaction that required ATP. After incubation of the BLM with reaction components for 2 h, >95% of the BLM was SUMOylated, and multiple moieties of SUMO-2 were attached to most of the BLM (**Figure 4A**). We then compared helicase activity of the SUMOylated and unSUMOylated BLM in two ways: (1) BLM SUMOylation reactions were prepared with or without ATP and reaction products were added directly to helicase assays; or, (2) SUMOylated and unSUMOylated BLM was partially purified from the SUMO reaction components by pull-down on nickel-NTA beads, after which bead-bound BLM was added to helicase assays. BLM activity was assayed on a ³²P-labeled DNA replication-fork-like substrate (38-nucleotide duplex DNA with two 12-nucleotide ssDNA tails) and percent unwinding was measured by electrophoresis through non-denaturing polyacrylamide gels followed by autoradiography. In both types of experiment, the percent of DNA substrate unwound over time was indistinguishable for SUMOylated compared to unSUMOylated BLM (**Figures 4B,C**). These results showed that BLM SUMOylation does not function to suppress BLM's intrinsic DNA helicase activity. Therefore, the excessive accumulation of fork-associated RPA in SM-BLM cells was not the result of failure to suppress BLM's intrinsic DNA helicase activity.

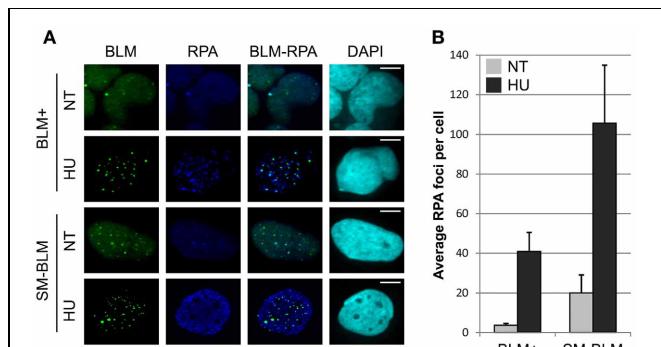


FIGURE 2 | Excess RPA accumulates at stalled replication forks in SUMO-mutant BLM cells. **(A)** Immunofluorescence images of BLM+ and SM-BLM cells untreated (NT) or treated with 0.5 mM HU for 24 h (HU) and stained for RPA. Bars indicate 10 μm. **(B)** Graphical representation of mean numbers of RPA foci. Three independent experiments were carried out for each of two BLM+ and two SM-BLM clones. Error bars represent the standard deviations of the results of each experiment. There were significantly more RPA foci in SM-BLM cells compared to BLM+ cells ($p < 0.001$) and HU treatment induced significantly more RPA foci in both cell lines ($p < 0.001$). Interaction was also observed indicating that the difference in effect of HU treatment on the two cell lines is significant ($p_{int} = 0.002$). P -values were calculated using mixed effects linear models as described in Materials and Methods.

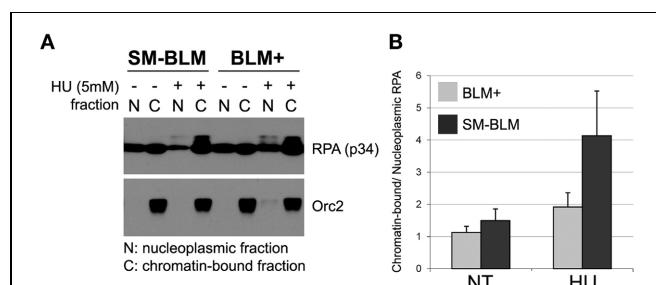


FIGURE 3 | Increased levels of RPA accumulate on chromatin in HU-treated SM-BLM cells. BLM+ and SM-BLM cells were untreated or treated with 5 mM for 6 h. **(A)** Chromatin and nucleoplasmic fractions were isolated from cells and solubilized RPA was analyzed by immunoblot using antibodies to the p34 subunit. Orc2 was used for quality control of the preparation of the chromatin-bound fraction. **(B)** Graph depicting the ratio of chromatin-bound RPA to nucleoplasmic RPA. A minimum of two independent experiments were carried out on each of two BLM+ and two SM-BLM clones. Error bars represent the standard deviations of the combined data.

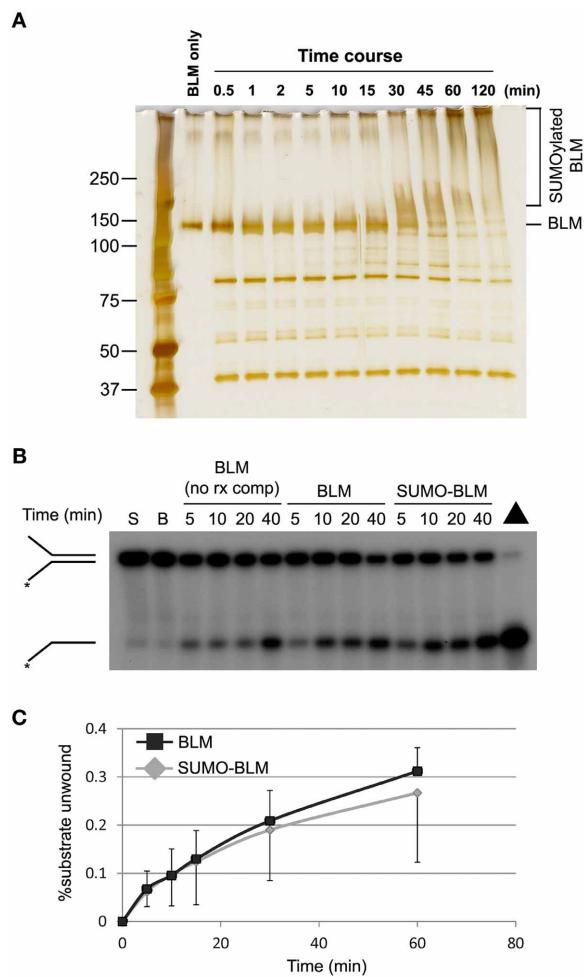


FIGURE 4 | DNA helicase activity of SUMOylated BLM is similar to unSUMOylated BLM. (A) BLM SUMOylation reactions were carried out, time points were removed, and the reactions were stopped with sample buffer. The reaction products were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis followed by silver staining. (B) SUMOylated (SUMO-BLM) or unSUMOylated BLM (BLM) was prepared by incubating 200 ng of purified recombinant BLM with SUMO reaction components in the presence (to make SUMOylated BLM) or absence (to make unSUMOylated BLM) of ATP. Helicase assays were performed by incubating the SUMO reaction products with 32 P-labeled synthetic replication-fork substrate, and the helicase reactions were stopped at different times. The products were analyzed by non-denaturing polyacrylamide gel electrophoresis followed by autoradiography. The position of the forked duplex and the unwound product are indicated on the left. S, unreacted substrate; B, beads only with unreacted substrate; Δ , boiled substrate; BLM (no rx comp), helicase reactions performed with BLM protein that was not exposed to SUMO reaction components as a positive control. (C) Graph showing quantification of the percentage of unwound substrate by SUMOylated and unSUMOylated BLM prepared in these experiments by nickel-NTA pull down. A minimum of two DNA unwinding experiments were performed for each method of BLM SUMOylation preparation shown in (B) and (C).

EXCESS BLM-RPA COMPLEX FORMS IN SM-BLM CELLS

BLM interacts with the 70 kD subunit of RPA (RPA-70), and RPA stimulates BLM's DNA helicase activity (Brosh et al., 2000). If

BLM SUMOylation functions to inhibit BLM-RPA interaction, then excess RPA accumulation in SM-BLM cells could result from a failure to suppress BLM-RPA interaction. To test this hypothesis, we compared the binding of purified RPA to SUMOylated or unSUMOylated BLM *in vitro*. BLM SUMOylation reactions were prepared with or without ATP, reaction products were mixed with RPA, and BLM-RPA complexes were pulled down with nickel-NTA beads. The amount of BLM-bound RPA was then measured by immunoblot analysis with anti-RPA/p70 antibodies. In these assays, the amount of BLM-bound RPA-70 detected was the same whether or not BLM was SUMOylated (Figure 5A). These data suggested that BLM SUMOylation does not directly affect interaction between BLM and RPA.

Because BLM SUMOylation could affect BLM and RPA interaction indirectly, we compared BLM-RPA complex formation *in vivo* by immunoprecipitation of BLM from HU-treated and untreated BLM+ vs. SM-BLM cells. In untreated cells, little to no detectable RPA was pulled down with BLM in either cell line. However, in HU-treated cells, more RPA was pulled down with BLM in SM-BLM cells compared to BLM+ cells (Figure 5B). Finding increased BLM-RPA complex formation in SM-BLM cells correlated with the excessive accumulation of RPA foci in SM-BLM cells, and it suggested that BLM SUMOylation has a role in preventing the accumulation of excess ssDNA at stalled forks.

To test whether the increased BLM-RPA complex formation in SM-BLM cells was trivially a consequence of higher levels of RPA expression in these cells, we performed immunoblot analysis of untreated and HU-treated SM-BLM and BLM+ cells. Analysis of total cell extracts indicated that SM-BLM and BLM+ cells contained similar amounts of RPA protein with or without treatment with HU (Figure 5C). Together with the *in vitro* data, the results suggested that BLM SUMOylation affects BLM-RPA complex formation indirectly, possibly through the mediation of other proteins at the fork. We concluded that BLM SUMOylation regulates BLM-RPA interaction at stalled forks.

In these experiments, we noted that HU-induced RPA phosphorylation did not differ significantly between BLM+ and SM-BLM cells, despite the presence of excess RPA at stalled forks in SM-BLM cells (Figure 5C).

DISCUSSION

The data presented here demonstrated that BLM SUMOylation regulates RPA and mediator accumulation at stalled replication forks, limiting the generation of excess ssDNA there. In HU-treated SM-BLM cells, a large excess of ssDNA accumulated at stalled forks, as evidenced by excess RPA foci and greater amounts of chromatin-bound RPA. With so much ssDNA generated at stalled forks in SM-BLM cells, we would expect large accumulations at the fork of both mediators BRCA2 and RAD52, and RAD51 as well. On the contrary, these proteins did not accumulate normally at stalled forks. These two observations are therefore evidence that BLM SUMOylation is necessary to license the HR mechanism at stalled forks, as mediator-dependent RAD51 complexes did not form on RPA-coated ssDNA in its absence. Because RAD51 loading onto ssDNA depends on mediator function, our results explain why RAD51 is not recruited to stalled

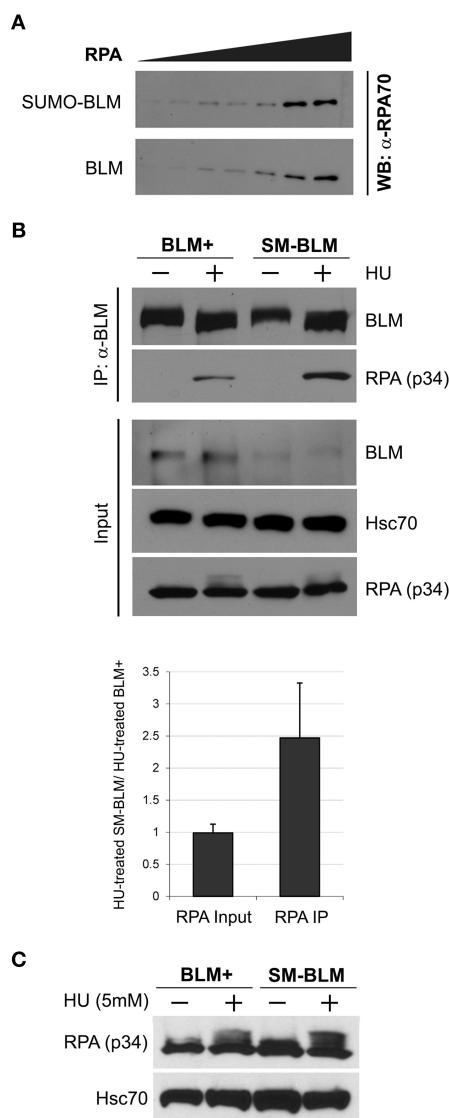


FIGURE 5 | Excess BLM-RPA complex forms in SM-BLM cells. (A) SUMOylation of BLM did not affect BLM-RPA binding *in vitro*. SUMOylated (SUMO-BLM) or unSUMOylated (BLM) BLM were bound to Ni-NTA beads and incubated with increasing amounts of purified human RPA protein for 30 min at room temperature. The beads were washed three times. Bound RPA protein was analyzed by immunoblot with anti-RPA/p70 antibody. A bead-only experiment showed that no excess RPA bound to beads in the absence of BLM. **(B)** BLM binds more RPA in SM-BLM cells than BLM+ cells. Cells were untreated or treated with 5 mM HU for 6 h. Immunoprecipitation of RPA was carried out using antibodies to the GFP tag on GFP-BLM. Input consisted of 2% of the initial cell extracts. Hsc70 was used as a loading control. A minimum of two independent experiments were carried out for each of two BLM+ and two SM-BLM clones. The graph depicts the ratio of input and immunoprecipitated RPA in HU-treated SM-BLM cells over HU-treated BLM+ cells. **(C)** HU does not induce significantly different levels of RPA and phosphorylated RPA in SM-BLM cells compared to BLM+ cells. Cells were untreated or treated with 5 mM HU for 6 h. Whole cell lysates were extracted and analyzed by immunoblot with antibodies to the p34 subunit of RPA. Hsc70 was used as a loading control. A minimum of two independent experiments were carried out for each of two BLM+ and of two SM-BLM clones. Error bars represent the standard deviations of the combined data.

forks in SM-BLM cells; however, the mechanism explaining why mediators are not recruited is still enigmatic.

The uncoupling of DNA replication from the replicative helicase during fork stalling can lead to extended stretches of ssDNA and later to replication-associated DSBs that could be processed by exonucleases (Petermann and Helleday, 2010). Because RPA-ssDNA complexes form on both of these cellular substrates, we do not know *a priori* whether BLM SUMOylation is important before or after the replication fork breaks. In our previous work (Ouyang et al., 2009), we showed that 24-h HU treatment of SM-BLM cells did not lead to increased DSBs in comparison to untreated SM-BLM cells. Moreover, the effect of BLM SUMOylation on RAD51 accumulation at stalled forks was apparent after a 1-h HU treatment of SM-BLM cells before DSBs have had a chance to accumulate (Saintigny et al., 2001; Petermann et al., 2010). Therefore, these data suggest that BLM SUMOylation is important in the generation of ssDNA after helicase-polymerase uncoupling and before fork breakage.

Formally speaking, the function of BLM SUMOylation in HR licensing at the fork could result from either positive or negative regulatory effects or both. Previous work has shown that RPA interacts with BLM, inhibiting BLM's strand annealing activity while increasing the efficiency of its helicase activity (Brosh et al., 2000; Doherty et al., 2005; Bartos et al., 2006). SUMOylation did not inhibit BLM-RPA interaction *in vitro*, yet more BLM-RPA complex formed in SM-BLM cells than in BLM+ cells after replication stress. Intriguingly, RPA is SUMOylated by SUMO-2 after treatment with camptothecin (CPT), which generates replication-associated DSBs (Dou et al., 2010). Cells that express a SUMO-mutant RPA have defects in RAD51 accumulation and DSB repair at IR-induced DSBs and CPT-induced broken forks; but increased RPA SUMOylation was not reported to occur during replication stress with UV or HU treatment (Dou et al., 2010). Thus, SUMOylation of BLM and RPA seem to have similar effects on the accumulation of RAD51, one in the context of replication fork stalling and the other in the context of replication fork breakage. Further studies are needed to examine the role of SUMOylation on the interaction of RPA and BLM and the possible effects of SUMOylation on each protein's biochemical activities.

In normal cells, the amount of ssDNA that accumulates after replication stalling is regulated by damage sensing and checkpoint pathways that recruit proteins to stabilize the fork and inhibit further unwinding (Sleeth et al., 2007). Because BLM protein is recruited to the fork immediately after stalling occurs, it is possible that pathologically unrestricted SM-BLM protein generates excessive DNA unwinding at the fork. We showed that BLM helicase activity is not inhibited by SUMOylation; therefore, if SUMOylation prevents BLM from unwinding DNA at the fork, it must do so via interaction with another protein. Because RPA stimulates BLM helicase activity, it is possible that SUMOylation negates RPA's stimulatory effect. Alternatively, BLM SUMOylation could have an indirect effect on the accumulation of RPA-ssDNA complex through interaction with ATR-mediated checkpoint signaling.

Another possibility is that BLM SUMOylation activates BLM or another protein's ssDNA annealing activity. A novel ssDNA

annealing function was recently identified in the N-terminal portion of BLM distinct from an annealing activity reported for the helicase domain (Chen and Brill, 2010); this region coincides with the region containing the SUMO-acceptor sites and the region required for SUMO binding (Eladad et al., 2005; Zhu et al., 2008). This ssDNA annealing activity of BLM could function to stabilize stalled forks by reannealing excess ssDNA. The annealing helicase HARP (HepA-related protein) has a role in ssDNA annealing that leads to stabilization of stalled forks (Bansbach et al., 2009; Driscoll and Cimprich, 2009; Yuan et al., 2009), and BLM SUMOylation could de-repress or directly promote the activities of these proteins (Yusufzai and Kadonaga, 2008, 2010; Sen et al., 2012).

Many key components and functions of the HR machinery are highly conserved. An exception to this rule lies with mediator proteins. While Rad52 is an essential component in yeast HR (Pâques and Haber, 1999), RAD52 gene knockout in mice shows no significant effect on cell viability or on HR and DNA repair capacities (Rijkers et al., 1998; Yamaguchi-Iwai et al., 1998). Instead, the BRCA2 mediator protein, a component that is not present in budding yeast, plays a central role in mammalian DSB repair by HR (Xia et al., 2001; Yang et al., 2005; Carreira et al., 2009). Recent work has shown that RAD52 inactivation is synthetically lethal with BRCA2 deficiency in human cell lines, indicating overlapping functions between RAD52 and BRCA2 in RAD51-dependent HR repair (Feng et al., 2011). Some have suggested that RAD52's primary function is not in the repair of DSBs with two broken ends but in the protection and repair of stalled and broken replication forks (Wray et al., 2008; Feng et al., 2011). Our results showed that SM-BLM cells have a defect in the accumulation of both RAD52 and BRCA2 at stalled forks, indicating that BLM SUMOylation is necessary for efficient mediator recruitment to stalled replication forks. We note that RAD52 is SUMOylated by the MMS21 E3 ligase, which is part of the SMC5/6 complex, and RAD52's SUMOylation is required for its normal repair function (Sacher et al., 2006; Santa Maria et al., 2007; Torres-Rosell et al., 2007; Ohuchi et al., 2008; Altmannova et al., 2010). Recent work has indicated that mediators, RAD51, and the Fanconi anemia complex have functions in replication fork stabilization that are independent of their roles in DSB repair (Schlacher et al., 2011, 2012; Feng and Zhang, 2012). RAD52 has ssDNA annealing activity that could help stabilize the replication fork (Wu et al., 2008; Grimme et al., 2010); consequently, the failure to recruit mediators to stalled forks in SM-BLM cells could explain the accumulation of excess RPA-ssDNA, if mediators are required for ssDNA annealing at the fork, or it could be an independent consequence of unSUMOylated BLM activity at the stalled fork.

While excess accumulation of RPA in HU-treated SM-BLM cells could result from either excessive DNA unwinding or impaired ssDNA re-annealing, there is yet another possible explanation relating to replication dynamics. Work in yeast and mammalian cells has shown that replication forks collapsed by prolonged replication stalling do not restart, and replication is rescued instead by new origin firing (Davies et al., 2007; Petermann et al., 2010). In SM-BLM cells, stalled replication forks may collapse more readily, leading to initiation of new forks to

compensate for the loss. In this model, the excess RPA foci would be accounted for by activation of dormant replication origins.

The SUMO pathway prevents aberrant recombination events from occurring at damaged replication forks. It positively regulates DSB repair by HR (Liberi et al., 2005; Branzei et al., 2006; Burgess et al., 2007) and it can influence repair pathway choice (Ulrich, 2009; Yang et al., 2011). Multiple components of the HR pathway are SUMOylated, but in most cases a complete mechanistic understanding of SUMO's role in functional regulation of its substrates is lacking. Protein-protein interactions between SUMO binding sites and SUMOs could be important in the recruitment of DNA repair factors such as 53BP1 and BRCA1 to the repair site (Bergink and Jentsch, 2009; Galanty et al., 2009; Morris et al., 2009). Recent work on the SUMO-targeted ubiquitin ligase RNF4 has suggested that SUMOylation could be important for dissociation of proteins such as MDC1 from the repair site (Galanty et al., 2012; Luo et al., 2012; Yin et al., 2012; Vyas et al., 2013), but ubiquitylation by RNF4 could also serve to recruit proteins with ubiquitin-binding sites by making hybrid SUMO-ubiquitin chains (Guzzo et al., 2012). From all these studies, SUMO seems to govern important turnover transitions, promulgating cycles of association and dissociation at the repair site: BLM SUMOylation could both assist in the recruitment of factors like RAD52 and RAD51 and it could also remove BLM from the repair site; both of these roles for SUMOylation would be absent in the SM-BLM protein.

BLM has many functions throughout the processes of DNA replication and HR repair. How these functions are regulated is an important question. SUMOylation is a dynamic process that confers diverse and unique roles to its substrate proteins. The present work shed light on how BLM SUMOylation regulates RPA and mediator functions at stalled forks, and more broadly, how replication forks are maintained under stress when HR is called into play. Our evidence suggests that BLM SUMOylation functions as a licensing mechanism that regulates and permits execution of the HR mechanism at damaged replication forks. The next step is to dissect the specific recruitment and turnover functions of SUMOylation on BLM and other proteins in this licensing mechanism.

MATERIALS AND METHODS

ANTIBODIES

For BLM immunoblot analysis, rabbit polyclonal anti-BLM antibodies raised against the first 431 amino acids of human BLM (Beresten et al., 1999) were used. For other immunoblot analyses, rabbit polyclonal anti- γ -H2AX antibodies (Abcam), mouse monoclonal anti-RPA/p34 antibody (Neomarkers), mouse monoclonal anti-RPA/p70 antibody (Neomarkers), rat monoclonal anti-ORC2 antibody (Abcam), and rat monoclonal anti-Hsc70 antibody (Assay Design) were used. Horseradish peroxidase-linked anti-mouse, anti-rabbit (Amersham), and anti-rat (Jackson ImmunoResearch) antibodies were used as secondary antibodies. Agarose conjugated rat monoclonal anti-GFP antibody D153-8 (MBL) was used for immunoprecipitation experiments. For indirect immunofluorescence, we used mouse monoclonal anti- γ -H2AX antibody (Upstate), rabbit polyclonal anti-RAD51 antibodies PC130 (Calbiochem), rabbit polyclonal

anti-RAD52 antibodies sc-8250 (Santa Cruz Biotechnology), mouse anti-BRCA2 antibodies 05-666 (Millipore), mouse monoclonal anti-RPA/p34 antibody (Neomarkers), Cy-5-labeled donkey anti-rabbit antibodies (Jackson ImmunoResearch), Alexa Fluor 594-labeled goat anti-mouse, Alexa Fluor 594-labeled goat anti-rabbit, and Alexa Fluor 647-labeled goat anti-mouse antibodies (Invitrogen).

IMMUNOFLUORESCENCE AND IMAGE ANALYSIS

The generation and characterization of BLM+ and SM-BLM cells was described previously (Eladad et al., 2005; Ouyang et al., 2009). Briefly, the SV40-transformed BS fibroblast cell line GM08505 was transfected with normal GFP-BLM or the SM-GFP BLM expression constructs, clones were isolated and selected for analysis that expressed similar amounts of GFP-BLM proteins. For indirect immunofluorescence, BLM+ and SM-BLM cells were seeded on coverslips and then treated with 0.5 mM HU in culture medium for 24 h. At the end of HU treatment, cells were washed and fixed and then stained with anti-RAD51, -BRCA2, -RAD52, or -RPA antibodies, and counterstained with appropriate secondary antibodies labeled with Alexa Fluor (Invitrogen). Fixation and staining was performed as described previously (Eladad et al., 2005; Ouyang et al., 2009). Coverslips were mounted with Prolong Gold antifade reagent containing 4',6-diamidino-2-phenylindole (DAPI; Invitrogen). Images were captured on a spinning disk confocal microscope (Carl Zeiss, LSM-510), and data were collected using Slidebook 4.1 software. Z-stacks were captured using a 100× oil immersion objective and the optical slice thickness was 0.2 μm. The BRCA2 data was collected on a laser-scanning confocal microscope (Carl Zeiss, LSM-710) and a single plane was imaged.

A focus was defined as a defined area of the nucleus greater than the minimum area of optical resolution ($>0.125 \mu\text{m}^2$) in at least one Z-stack in which the fluorescence intensity was greater than the background fluorescence intensity of the nucleoplasm. The maximum number of foci that could be counted in these cells was 150. A typical immunofluorescence experiment consisted of assessment of 30–50 cells per condition. The data presented are from two to three independent experiments performed on two to three clones of each type. Immunofluorescence images for figures were created using Image J and Metamorph software (Molecular Devices).

IMMUNOBLOT ANALYSES

Proteins from cell lysates, chromatin, and nucleoplasmic fractions, and immunoprecipitates were separated on 4–15% sodium dodecyl sulphate (SDS)/polyacrylamide gradient gels (Bio-Rad) and transferred on to polyvinylidene fluoride membranes (Bio-Rad). The membranes were blocked for 1 h at room temperature with Tris-buffered saline (Boston Bioproducts) containing 0.1% Tween 20 and 5% powdered milk (Bio-Rad), washed, and subsequently probed with appropriate primary antibodies either for 2 h at room temperature or overnight at 4°C. The membranes were then incubated with the appropriate horseradish peroxidase-linked secondary antibodies for 1 h at room temperature, washed, and incubated with Western Lightning-ECL, Enhanced Chemiluminescence reagent (Perkin Elmer) for 5 min at room temperature. Proteins labeled with antibodies on the membrane were visualized by detection on film.

CHROMATIN ISOLATION

Chromatin preparations were made by the method of Méndez and Stillman (2000). Briefly, cells treated with or without 5 mM HU for 6 h were harvested by centrifugation, washed in PBS, and resuspended in buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.34 M sucrose, 10% glycerol, 1 mM DTT, 0.1 mM PMSF, and protease inhibitors cocktail). Triton X-100 (0.1%) was added, and the cells were incubated for 5 min on ice. Nuclei were collected in the pellet by low-speed centrifugation at 1300 × g for 4 min at 4°C. Nuclei were washed once in buffer A, and then lysed in buffer B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, protease inhibitors cocktail). Nucleoplasmic proteins were separated from chromatin-bound proteins by centrifugation at 1700 × g for 5 min at 4°C. Nucleoplasmic fractions were collected in the supernatant. The chromatin pellet was washed once in buffer B, and centrifuged again under the same conditions. The final chromatin pellet was resuspended in Laemmli sample buffer. HU treatment was performed at 5 mM for 6 h so that BLM quantities would be nearly the same in untreated and treated cells. With respect to the phenotypes studied here, there are no major differences between the 6- and 24-h treatments (Ouyang et al., 2009).

IMMUNOPRECIPITATION

Cells treated with or without 5 mM HU for 6 h were harvested, washed twice in PBS buffer, and resuspended in NP-40 lysis buffer (Boston Bioproducts) containing protease inhibitors (Roche) for 30 min. Cell lysates were cleared by centrifugation (15 min, 14,000 × g, 4°C). Cleared cell lysates were then incubated with 20 μl of agarose beads conjugated with anti-GFP antibodies and allowed to mix overnight at 4°C. Proteins bound to beads were separated from the supernatant by centrifugation (3 min, 2000 × g, 4°C) and washed with 5 times volume of lysis buffer. Proteins were extracted from the beads by boiling in Laemmli buffer (Boston Bioproducts) for 5 min and subsequently analyzed by immunoblot.

SUMOYLATION OF BLM

Two hundred nanograms of His-tagged full-length BLM, purified according to the method of Karow et al. (1997), was SUMOylated in a 50 μl reaction containing 10 mM HEPES (pH 7.3), 110 mM potassium acetate, 2 mM MgCl₂, 2 mM ATP, and purified recombinant proteins (Zhu et al., 2008), including 200 ng E1, 100 ng UBC9, and 200 ng SUMO-2, at 37°C for increasing amounts of time (0.5, 1, 2, 5, 10, 15, 30, 45, 60, and 120 min). Reactions were stopped by the addition of sample buffer and subsequently analyzed by SDS-polyacrylamide gel electrophoresis followed by Silver staining (Pierce Silver Stain Kit, Thermo Scientific) per the manufacturer's instructions.

DNA SUBSTRATE

Oligonucleotides for the fork substrate designed according to Mohaghegh et al. (2001) were purchased from Integrated DNA Technologies. A single oligonucleotide was 5'-end-labeled with [γ -³²P]ATP (PerkinElmer Life Sciences) using T4 polynucleotide kinase in PNK buffer at 37°C for 30 min per the manufacturer's instructions (New England Biolabs). The labeled substrate was purified using QIAquick Nucleotide Removal kit (Qiagen). The [γ -³²P]ATP-labeled oligonucleotide was then annealed

with a two fold excess of the unlabeled complementary oligonucleotide in annealing buffer (40 mM Tris-HCl, pH 8.0, 50 mM NaCl) by heating at 95°C for 5 min and then cooling slowly to room temperature.

HELICASE ASSAY

Equal amounts of SUMOylated BLM and unSUMOylated BLM, respectively, were added to 100 µl of nickel-NTA bead slurry (Qiagen) that had been blocked in 0.5% BSA for 2 h at room temperature and then washed two times in BLM binding buffer (60 mM Tris-HCl, pH 7.4, 20 mM KCl). BLM was bound to beads for 1 h at room temperature and washed two times in BLM binding buffer. Helicase reactions were performed with 20 ng BLM in 10 mM Tris-HCl (pH 7.5), 4 mM MgCl₂, 1 mM dithiothreitol, 2 mM ATP, 0.1 mg/ml BSA, and 0.3 nM of labeled helicase substrate at 37°C for either 5, 10, 15, 20, 30, 40, or 60 min. Helicase reactions were stopped by adding gel loading dye (50 mM EDTA, 40% glycerol, 0.9% SDS, 0.05% bromophenol blue, and 0.05% xylene cyanol). Reaction products were analyzed by electrophoresis through pre-cast 10% non-denaturing polyacrylamide gels (Bio-Rad). The amount of substrate unwound was measured by autoradiography using a phosphorimager (Molecular Dynamics).

In vitro BINDING ASSAY

Two hundred nanograms of SUMOylated and unSUMOylated BLM, respectively, were added to 200 µl of Ni-NTA bead slurry (Qiagen) that had been previously incubated in Blocking Buffer (PBS, 0.5% Tween 20, 3% BSA) for 2 h at room temperature and subsequently washed three times with PBS. Increasing concentrations of purified RPA were then incubated with 20 ng of either SUMOylated BLM or unSUMOylated BLM bound to nickel-NTA beads, respectively, in binding Buffer (50 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 5 mM ATP, 100 µg/ml BSA, and 50 mM NaCl) for 30 min at room temperature. Beads were then washed three times in binding buffer. The amount of RPA-70 protein bound to SUMOylated BLM

or unSUMOylated BLM was analyzed by immunoblot with anti-RPA/p70 antibodies.

STATISTICAL ANALYSIS

Because observations within each clone may be correlated, we used mixed effects linear models to test the data for statistical significance. In the mixed effects models, each clone was treated as a random effect and the experimental variables were treated as fixed effects. For testing changes in the number of foci/cell, cell type (BLM or SM), treatment (with and without HU), and interaction terms for cell type by treatment were treated as fixed effects. Because the foci data were not normally distributed, we used a generalized estimating equation approach that can account for non-normal correlated data with non-homogeneous variances.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: frontiersin.org/Cancer_Genetics/10.3389/fgen.2013.00167/abstract

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Chromatin modifications and DNA repair: beyond double-strand breaks

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DNA repair must take place in the context of chromatin, and chromatin modifications and DNA repair are intimately linked. The study of double-strand break repair has revealed numerous histone modifications that occur after induction of a DSB, and modification of the repair factors themselves can also occur. In some cases the function of the modification is at least partially understood, but in many cases it is not yet clear. Although DSB repair is a crucial activity for cell survival, DSBs account for only a small percentage of the DNA lesions that occur over the lifetime of a cell. Repair of single-strand gaps, nicks, stalled forks, alternative DNA structures, and base lesions must also occur in a chromatin context. There is increasing evidence that these repair pathways are also regulated by histone modifications and chromatin remodeling. In this review, we will summarize the current state of knowledge of chromatin modifications that occur during non-DSB repair, highlighting similarities and differences to DSB repair as well as remaining questions.

Keywords: histone modification, chromatin remodeler, DNA structure, sister chromatid recombination, gap repair, stalled replication fork, excision repair, mismatch repair

INTRODUCTION

Assaults to the genome are common throughout the lifetime of a cell and DNA damage can occur by endogenous factors, such as reactive oxygen species, base mismatches, and alternative (non-B form) DNA structures, or exogenous factors, such as ultraviolet (UV) radiation and environmental toxins. At the occurrence of a DNA lesion, the cell will initiate repair to protect the integrity of the genetic material. As the genome is condensed into chromatin, repair must work within the context of the chromatin structure to access and repair the damaged DNA.

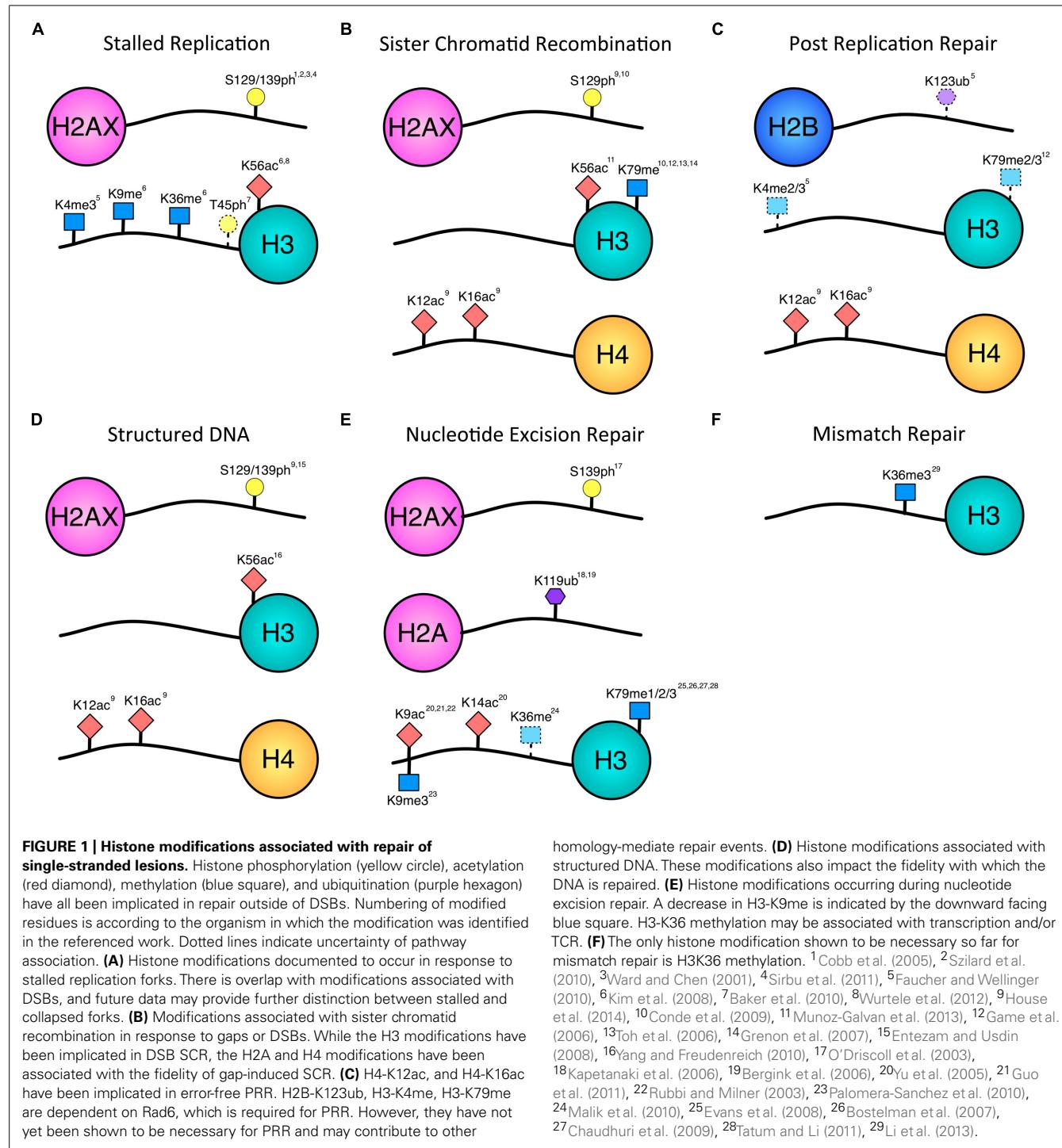
One mechanism to alter chromatin structure is to modify histone residues by the addition of chemical groups such as a phosphate, acetyl, or one or more methyl groups. Small peptides such as ubiquitin and SUMO can also be added to lysine residues. These histone modifications can change nucleosome-DNA or nucleosome-nucleosome interactions to either open or condense the chromatin structure. For example, acetylation of histone H4 at lysine 16 (H4K16ac) reduces the interaction between the H4 tail and the H2A acidic pocket, inhibiting higher-order nucleosomal folding and resulting in a more open chromatin confirmation (Shogren-Knaak et al., 2006; Robinson et al., 2008). Alternatively, histone modifications that occur upon DNA damage can alter the interaction of non-histone proteins with chromatin to facilitate direct recruitment of repair factors and contribute to checkpoint initiation and termination (Humpal et al., 2009). ATP-dependent chromatin remodelers are also actively altering the chromatin landscape to promote repair (Seeber et al., 2013). Remodelers can slide nucleosomes, evict whole or partial nucleosomes, or alter the interaction between nucleosomes and DNA (Seeber et al., 2013). Still, in many cases, the details of how histone modifications and chromatin remodeling are affecting the formation, or progression of repair intermediates

is not well understood. These intermediates include replication fork stabilization, strand resection, gap filling, and strand invasion or extension. The efficiency of formation or resolution of repair intermediates could ultimately dictate repair-pathway choice.

DNA double-strand breaks (DSBs) are considered to be the most lethal type of DNA damage and the chromatin factors mediating repair of these lesions have been extensively studied. However, DSBs are rare, and more common threats to the genome include single-strand DNA gaps, nicks, base lesions, stalled replication forks, and non-canonical DNA topology that can interfere with replication and repair. The chromatin modifications that are occurring during these other types of DNA repair pathways remain less well-characterized than DSB repair because of the technical difficulty associated with studying a site-specific, non-DSB lesion compared to robust DSB-inducing systems. However, studies have started to elucidate the contribution of histone modifications to non-DSB lesions (Figure 1). This review will focus on the chromatin modifications known to date to contribute to repair of single-strand DNA gaps, stalled forks, DNA structures, base lesions and mismatches, and will compare and contrast these marks to those known to occur during DSB repair.

CHROMATIN MODIFICATIONS AND REMODELING DURING DSB REPAIR

At the occurrence of a DSB, the MRN (MRX in yeast) complex binds the broken DNA ends, which recruits the PIKK kinases, ATR/ATM (Mec1/Tel1 in yeast); alternatively, the broken ends are bound by the Ku70/Ku80 heterodimer, which recruits DNA-PK (Gottlieb and Jackson, 1993; Mahaney et al., 2009). These kinases phosphorylate H2AX, thus forming γH2AX and initiating the chromatin response to DNA



damage (Rogakou et al., 1998; Downs et al., 2004; Ataian and Krebs, 2006; Bao, 2011). The histone modifications documented to occur during DSB repair will be briefly summarized here in order to provide context for comparison to other repair pathways. For recent, more detailed reviews, see Chubb and Rea (2010), Zhu and Wani (2010), Bao (2011), Greenberg (2011), Miller and Jackson (2012), Seeber et al. (2013), Price and D’Andrea (2013), and Tsabar and Haber (2013).

γ H2AX AT DSBs

γ H2AX (H2AX-S139ph in mammals; H2A-S129ph in yeast) is the most well-documented histone modification in response to a DNA DSB and occurs within minutes of break induction (Rogakou et al., 1998; Shroff et al., 2004; Stiff et al., 2004, 2006). The γ H2AX domains are established by a positive feedback loop whereby γ H2AX recruits the mammalian repair mediator MDC1, which in turn recruits additional MRN that will stimulate further

phosphorylation of H2AX by ATM (Uziel et al., 2003; Lukas et al., 2004; Lee and Paull, 2005; Stucki et al., 2005; Lou et al., 2006). This MDC1-ATM pathway to amplify the γ H2AX signal increases the density of γ H2AX proximal to the break site; however, subsequent spreading of γ H2AX to create large domains is dependent on the action of ATM, but not MDC1 (Savic et al., 2009). Earlier data in yeast indicated that the γ H2AX modification is necessary for the recruitment of chromatin modifying enzymes, including INO80 and SWR1 remodelers (Downs et al., 2004; Morrison et al., 2004; van Attikum et al., 2004), which can alter the chromatin structure to allow access by other repair proteins, such as 53BP1, Rad51, and BRCA1 (Paull et al., 2000; Celeste et al., 2003a,b; Murr et al., 2006; Shi and Oberdoerffer, 2012; Scully and Xie, 2013). However, recent work in yeast demonstrated that the γ H2AX modification is dispensable for recruitment of the chromatin modifying enzymes INO80, SWR-C, NuA4, SWI/SNF, or RSC to DSBs during homologous recombination in G2 cells (Bennett et al., 2013), suggesting that the repair proteins are not necessarily directly recruited by an interaction with γ H2AX. Instead, recruitment of chromatin modifiers and remodelers in G2 is tightly coupled to homologous recombination, and the Rad51 filament itself may play a role (Bennett et al., 2013). In any case, γ H2AX modification is an early step in a cascade of chromatin modifications, including nucleosome remodeling and other post-translational histone modifications, which allows for subsequent recruitment and retention of repair factors. Although many histone modifications that contribute to DSB repair have been identified, the order of events is only partially understood (Bao, 2011).

In addition to γ H2AX, other histone modifications are required for efficient repair of DSBs, including acetylation, methylation, and ubiquitination of lysine residues. The modification of amino acid residues can be influenced by existing histone modifications. For example, H4-S1 phosphorylation after DNA damage is required for H4 N-tail lysine deacetylation (Cheung et al., 2005; Utley et al., 2005), and H2B ubiquitination is required for H3 methylation (Game and Chernikova, 2009; discussed below). This presents the interesting possibility that ordered progression of modifications could allow for regulation of repair events or be important in promoting proper repair.

HISTONE METHYLATION

Defective methylation of H3-K79 and H3-K36 results in ionizing radiation (IR) sensitivity in yeast cells (Game et al., 2005, 2006; Grenon et al., 2007; Game and Chernikova, 2009). In mammalian cells, H3-K79 methylation, along with H4-K20 dimethylation, are recognized by 53BP1 in relaxed chromatin at the DSB (Hartlerode et al., 2012; Wakeman et al., 2012; Hsiao and Mizzen, 2013). H3-K9me3, on the other hand, stimulates TIP60 histone acetyltransferase (HAT) activity at the break site, resulting in acetylation of both histones and ATM, the latter activating the kinase to further stimulate γ H2AX formation (Ikura et al., 2000; Murr et al., 2006; Xu et al., 2010, 2012; Bao, 2011; Xu and Price, 2011).

HISTONE UBIQUITINATION

Histone ubiquitination has been implicated in several steps of DSB repair (Bao, 2011). H2AX-K119 ubiquitination is induced upon IR treatment (Xie et al., 2010) and is required for histone

turnover at the site of damage (Ikura et al., 2007). H2A/H2A.X ubiquitination by RNF8 and RNF168 is also required for accumulation and retention of 53BP1 and BRCA1 at the break (Huen et al., 2007; Kolas et al., 2007; Mailand et al., 2007; Doil et al., 2009). H3 and H4 ubiquitination have also been shown to facilitate the recruitment of repair factors to a DSB, and in mammalian cells monoubiquitination of H2B-K120 is required for recruitment of both HR and NHEJ repair factors and may contribute to chromatin decompaction to promote repair (Wang et al., 2006; Moyal et al., 2011). In yeast, ubiquitination of H2B-K123 is a prerequisite for H3-K4 and H3-K79 methylation and is necessary for Rad53 phosphorylation in response to DNA damage (Giannattasio et al., 2005).

HISTONE ACETYLATION AND DEACETYLATION

Histone acetylation flanking a DSB is required for repair and cellular survival after DNA damage in both yeast and mammalian cells (Bird et al., 2002; Downs et al., 2004; Tamburini and Tyler, 2005; Murr et al., 2006; Xu et al., 2010). In yeast, histone lysine residues are acetylated at DSB sites by Gnc5, an H3-specific HAT recruited by γ H2AX (Lee et al., 2010); in mammalian cells, histones are acetylated by TIP60, the NuA4 complex HAT that is recruited to a DSB by a physical interaction with the MRN complex (Chailleux et al., 2010). An additional mammalian HAT, MOF, acetylates histone H4-K16 and this modification is required for the recruitment of repair factors to an irradiation-induced break site, including MDC1, 53BP1, and Brca1 (Li et al., 2010; Krishnan et al., 2011).

As histone modifications are required to alter the chromatin environment to facilitate repair, additional modifications are required to reset the chromatin state once repair is complete. Histone deacetylases (HDACs) are recruited to remove histone acetyl marks and restore the chromatin structure in yeast (Tamburini and Tyler, 2005). However, HDACs may also play a more direct role in the repair process, as in mammalian cells HDACs are recruited to a DSB early in the repair process (Bao, 2011; Xu and Price, 2011). In mammalian cells, the HDAC SIRT1 is recruited to an I-SceI DSB (Oberdoerffer et al., 2008), and the HDAC complex NuRD, which includes HDAC1 and HDAC2, is recruited to a microirradiation-induced DSB to deacetylate H3-K56 (Miller et al., 2010), a histone modification that promotes nucleosome assembly during replication and repair (Chen et al., 2008; Li et al., 2008).

CHROMATIN REMODELERS

Chromatin remodelers have also been shown to play an important role in DSB repair. Interestingly, the NuRD HDAC complex contains a chromatin remodeler subunit (MTA1 or 2), and the NuA4 HAT complex contains the chromatin remodeler p400 (Xu and Price, 2011; Price and D'Andrea, 2013), intimately linking the role of histone modifications and chromatin remodeling. p400 (SWR1 in yeast) has recently been shown to catalyze the exchange of the H2A variant H2A.Z onto the chromatin at DSBs, which leads to a more open chromatin structure and promotes further histone modifications at the site of damage (Xu et al., 2012). Once at the site of damage, SWR1 stimulates the exchange of H2A.Z onto the chromatin and this exchange is promoted by both H2A and H4

acetylation (Altaf et al., 2010). In yeast, the direction of exchange (H2AX for H2A.Z or H2A.Z for H2AX) is influenced by H3-K56 acetylation state: SWR-C preferentially removes H2A.Z from the nucleosome when H3 is acetylated at K56 (H3-K56Q acetyl-mimic mutant), and thus the specific catalytic activity of the SWR-C remodeler is determined by histone modification state to promote turnover of histone variants (Watanabe et al., 2013). SWR1 also facilitates Ku80 binding at the break, thereby promoting NHEJ (van Attikum et al., 2007; Bao, 2011).

Chromatin remodelers appear to play a key role during repair, but the exact function of many remodelers remains unknown. What DNA topological substrate is specifying remodeler recruitment or action and how histone modifications contribute to this process or remodeler function remain to be elucidated. Chromatin remodelers may be required during repair to open the damaged DNA to other repair proteins; alternatively, remodelers could be important to downregulate transcription in the vicinity of the break to limit collisions between the repair machinery and transcription machinery, allowing repair to progress properly (Kruhlak et al., 2007; Shanbhag et al., 2010). More generally, a transient repressive chromatin state may be important for stabilization of the chromatin fiber for efficient repair, as was a proposed role for H3-K9me3 at a DSB (Ayrapetov et al., 2014). In addition to physically altering the chromatin to facilitate proper access to the DNA template, remodelers could be more directly involved in the subsequent cascade of damage signaling by directly interacting with other repair factors, perhaps acting as recruitment platforms or mediators.

CHROMATIN MODIFICATIONS ASSOCIATED WITH STALLED REPLICATION FORKS

Stalled replication forks can be protective to genomic integrity, given that the stall can avoid replication through damaged DNA and signal the location of DNA damage to be repaired. However, if the damage is not repaired or bypassed, or if a single-strand break is in the template, the stalled fork can collapse, leading to a DSB. For example, low doses of aphidicolin can induce replication stress that will stall forks and leave single-strand gaps, eventually resulting in DSBs (Freudenreich, 2007). Interestingly, aphidicolin treatment during S phase induces γH2AX-dependent 53BP1 foci in the next G₁ phase, indicating that a fork stall not resolved by mitosis can lead to a DSB in the next cell cycle (Harrigan et al., 2011; Lukas et al., 2011).

To prevent DSB formation, damage tolerance pathways can be invoked, leaving the damage to be resolved through post-replication repair (PRR). Error-prone PRR occurs by recruitment of a translesion synthesis (TLS) polymerase that can bypass the lesion. Alternatively, a template switch involving sister chromatid annealing can allow the polymerase to copy the homologous sequence information from the sister chromatid and continue replication, or sister chromatid recombination (SCR) can be used to repair a gap left after fork passage. Thus, since fork stalling can lead to TLS, template switching, SCR, or a DSB, it can be experimentally difficult to distinguish the chromatin modifications that are specific to the initial fork stall or to each subsequent repair pathway. Stalling replication forks with low levels of hydroxyurea (HU) or inducing site-specific stalls with

DNA-bound proteins or known fork-stalling DNA sequences, such as CGG repeats, can be effective strategies to uncover chromatin modifications associated with stalled replication forks. In addition, co-localization experiments using ChIP or isolation of proteins on nascent DNA (iPOND, Sirbu et al., 2012; see below) have been productive in linking replication fork-associated factors with chromatin-associated factors. This section will focus on the histone modifications and chromatin remodelers known to-date to be associated with stalled replication forks.

Stalled replication forks are marked in the chromatin as DNA damage, as γH2AX domains form at stalled replication forks (**Figure 1A**). In yeast, phosphorylated H2A (γH2A) was found to co-localize with HU stalled forks and Pol ε by ChIP, and this event was dependent on the Mec1 but not the Tel1 kinase, distinguishing the modification from γH2A at a DSB which can be phosphorylated by both Mec1 and Tel1 (Cobb et al., 2005). Indeed, genome-wide mapping of γH2A-rich loci using ChIP technology revealed that γH2A is enriched at sites of natural replication fork stalling, including the rDNA locus, tRNA genes, LTRs, telomeres, and DNA replication origins (Szilard et al., 2010). Interestingly, the average size of the γH2A domain at these natural pause sites was 1255 bp, in contrast to the 50 kb domain detected at an HO endonuclease-induced DSB in yeast cells. Functionally, H2A modification is required to promote replication fork progression, as measured by total DNA content after release from G₁ in *mec1-ts* mutants, and prevent DSB formation, as measured by pulsed-field gel electrophoresis (Cha and Kleckner, 2002).

In mammalian cells, γH2AX is induced when DNA replication is inhibited by HU, forming foci that co-localize with PCNA in S phase cells, and this response is also dependent on ATR but not ATM (Ward and Chen, 2001). Using iPOND technology to monitor protein dynamics at sites of newly synthesized DNA in live mammalian cells, γH2AX was detected at a stalled replication fork within 10 min after HU addition, becoming maximal at 30 min (Sirbu et al., 2011). These early time points were not accompanied by markers of DSBs such as Mre11, DNA-PK, or Ku70/Ku80 which appeared at later time points 2–4 h after HU addition, indicating that the early γH2AX is not marking collapsed forks or DSBs. The γH2AX domain spread from the site of fork stalling over time, reaching tens of thousands of base pairs by 1 h. Again, initial γH2AX formation at an HU-stalled replication fork was ATR-dependent; but maintenance of the γH2AX domain at later time points was ATM-dependent, likely occurring once the persistently stalled fork had collapsed into a DSB (Sirbu et al., 2011).

While both DSBs and stalled forks are marked by an initial γH2AX histone modification, subsequent chromatin modifications dependent on either ATM or ATR could produce chromatin environments specific to the lesion type, directing repair to the appropriate pathway or influencing the repair process itself. The histone modifications important for turning off the DNA damage response at a stalled fork may also be different than at a DSB. To turn off the DSB-induced checkpoint, mammalian serine/threonine phosphatase complexes PP2A and PP4 and the yeast PP4C ortholog Pph3 dephosphorylate γH2AX, leading to inactivation of Rad53 (Chowdhury et al., 2005; Keogh et al., 2006; Nakada

et al., 2008). However, another phosphatase, PP1 (Glc7), has been shown to dephosphorylate γH2AX and contribute to Rad53 inactivation and replication fork restart after HU treatment (Bazzi et al., 2010). Of note, PP4 in mammalian cells appears to be especially important for resolution of DNA damage that occurs during replication, specifically dephosphorylating ATR (but not ATM)-modified γH2AX (Chowdhury et al., 2008).

Although much research has focused on γH2AX at stalled replication forks, other histone modifications are likely occurring to influence replication fork recovery or repair (**Figure 1A**). One such modification is phosphorylation of H3-T45 in yeast, a modification observed in response to prolonged replication stress in HU treated cells that is independent of the Mec1 and Tel1 kinases and is instead regulated by the Cdc7-Dbf4 kinase complex (Baker et al., 2010). The authors conclude that this modification is specific to replication stress, as treatment with DNA alkylating agent MMS did not increase H3-T45 phosphorylation. Further, the H3-T45A mutant was not sensitive to MMS, but was sensitive to HU and CPT, a topoisomerase I inhibitor, as measured by cell survival in a spot assay (Baker et al., 2010). However, prolonged exposure to HU and CPT will lead to DSB formation and therefore this modification could mark DSBs, although in a Mec1/Tel1-independent manner.

In addition to histone phosphorylation, histone acetylation, methylation, and ubiquitination likely play a role in signaling replication stress (**Figure 1A**). In human T-cell lymphoma cells, HDAC3 is localized to replication forks by iPOND, linking changing acetylation state with newly synthesized DNA (Wells et al., 2013). Further, HDAC3 inhibition resulted in decreased replication fork velocity and increased apoptosis that was associated with increased DNA damage and an S phase defect (Wells et al., 2013). In budding yeast, H3-K56 acetylation is required to complete replication in the presence of lesions caused by MMS (Wurtele et al., 2012). In fission yeast, the absence of Clr4 and Set2, the methyltransferases for H3-K9 and H3-K36, respectively, leads to a decrease in HU-induced phosphorylation of Cdc2 and Mik1, downstream actors in the Rad3 (human ATM) checkpoint pathway. Therefore, the authors conclude that the HU replication stress checkpoint requires H3 methylation by Clr4 and Set2 (Kim et al., 2008). H3-K4 trimethylation may also contribute to repair of S phase damage in *S. cerevisiae*, as the absence of Set1, the HMT responsible for H3-K4 trimethylation, leads to an S phase progression defect, in addition to the role of Set1 in NHEJ (Faucher and Wellinger, 2010). Histone H3-K4 and K79 methylation, regulated by H2B-123 ubiquitination, may also play a role in PRR, which would be initiated after a replication fork stalling event (discussed in next section).

Chromatin remodeling is also important in resolving the damage at a stalled replication fork (**Table 1**). INO80 is implicated in recovery from stalled replication in both budding yeast and mammalian cells. In mammalian cells, *ino80* mutants are HU sensitive, display defective S phase progression, and are defective in recovery from replication stress (Hur et al., 2010; Min et al., 2013; Vassileva et al., 2014). In yeast, Ino80 is enriched at stalled replication forks, as detected by ChIP (Papamichos-Chronakis and Peterson, 2008; Shimada et al., 2008). In addition, recovery

from replication fork stalling after HU treatment is impaired in an *ino80* mutant, resulting in DSBs (Shimada et al., 2008), and Ino80 promotes replication restart after MMS treatment (Falbo et al., 2009). In the absence of both an intact INO80 complex and the chromatin remodeler Isw2, recovery from the S phase checkpoint response is defective (Au et al., 2011). The chromatin remodeler RSC2 may also play a role in recovery from stalled replication or be involved in PRR. In *S. cerevisiae*, RSC2 is found near replication forks by ChIP, and PCNA ubiquitination is significantly decreased in a *rsc2Δ* mutant after MMS, UV, and HU treatments (Niimi et al., 2012). Similarly, depletion of the human homolog BAF180 of the PBAF complex led to a decrease in fork progression by IdU incorporation (DNA fiber) analysis and decreased chromatin bound unmodified and ubiquitin-modified PCNA and Rad18 (Niimi et al., 2012). Other remodelers are found at replicating forks irrespective of a stall, but may also play a role at stalled forks (Vincent et al., 2008; Au et al., 2011; Bhaskara et al., 2013).

It is likely that additional histone modifications are associated with recovery from stalled replication forks, but they remain to be identified. Histone modifications could influence several steps of recovery from stalled replication, including marking the location of a stalled fork, recruitment of replication restart factors or replication bypass factors (including translesion synthesis polymerases), establishing sister chromatid cohesion for homology-mediated PRR, and finally the recruitment of chromatin modifying enzymes to reset the chromatin structure.

CHROMATIN MODIFICATIONS IN RESPONSE TO SINGLE-STRAND GAPS REPAIRED BY SISTER CHROMATID RECOMBINATION OR TEMPLATE SWITCHING

More common than DSBs are single strand DNA lesions that can occur during replication and repair. Single strand gaps that occur during replication will activate the DNA damage checkpoint (Branzei and Foiani, 2008). These gaps activate the kinase ATR, not ATM, and the intensity of the checkpoint response increases with increasing gap length, as monitored by Chk1 phosphorylation (MacDougall et al., 2007). To prevent gaps from becoming DSBs, Rad6-Rad18 dependent damage tolerant replication can be invoked to allow replication to continue, followed by subsequent repair of the template-strand lesion in a process that has been termed PRR. Single-stranded gaps that occur during replication must be resolved before the following S phase to prevent the formation of DSBs. Base damage, for example by alkylating agents such as MMS, result in unreplicated gaps left after fork passage (Hashimoto et al., 2010). It is probable that repair of nicks and gaps will have overlapping histone modifications with DSB repair, particularly if the lesion induces a checkpoint response. However, it is also likely that different combinations of histone modifications will distinguish nick and gap repair pathways from repair of a DSB.

POST-REPLICATION REPAIR

Post-replication repair can be divided into two Rad6-dependent, damage tolerant pathways: error-prone TLS and error-free PRR.

Table 1 | Chromatin remodelers associated with repair pathways outside of DSB repair.

Repair pathway	Remodeling complex	Implicated subunit	System	Reference
Stalled Replication	INO80	Ino80	Yeast	Papamichos-Chronakis and Peterson (2008), Shimada et al. (2008), Falbo et al. (2009)
			Human	Hur et al. (2010), Vassileva et al. (2014)
			Mouse	Min et al. (2013)
	ISW2	Iszw2	Yeast	Vincent et al. (2008), Au et al. (2011)
SCR	RSC	Rsc2	Yeast	Niimi et al. (2012)
		BAF180	Human cells	Niimi et al. (2012)
		Rsc1	Yeast	House et al. (2014)
PRR	RSC	Rsc2	Yeast	Baetz et al. (2004), Oum et al. (2011), House et al. (2014)
		Rsc7	Yeast	Oum et al. (2011)
		Rsc1	Yeast	Niimi et al. (2012), House et al. (2014)
Structured DNA	RSC	Rsc2	Yeast	House et al. (2014)
		SWR1	Yeast	House et al. (2014)
	SWI/SNF	Snf2	Yeast	House et al. (2014)
GRG	SWI/SNF	Snf5	Yeast	Yu et al. (2005)
			Human cells	Gong et al. (2006)
				Ray et al. (2009), Zhao et al. (2009), Zhang et al. (2009)
		Snf6	Yeast	Gong et al. (2006)
		BRG1	Human cells	Zhao et al. (2009), Zhang et al. (2009)
		SWI/SNF-like	Rad16	Ramsey et al. (2004), Yu et al. (2011)
			ALC1	Pines et al. (2012)
		INO80	Ino80	Sarkar et al. (2010)
			Ino80, Arp5	Jiang et al. (2010)
		ISWI	ACF	<i>in vitro</i> (<i>Drosophila</i>)
TCR	SWI/SNF-like	CSB	<i>in vitro</i> (Human)	Citterio et al. (2000)
		Rad26	Yeast	Gregory and Sweder (2001)
	ISW1	SMARCA5/SNF2H, WSTF, ACF	Human cells	Aydin et al. (2014)
	ISW1, ISW2	Iszw1, Iszw2	<i>in vitro</i> (yeast)	Nakanishi et al. (2007)
BER	RSC	Sth1	Yeast	Czaja et al. (2014)
	SWI/SNF	Complex	<i>in vitro</i> (yeast)	Menoni et al. (2007)

TLS is initiated by Rad6-Rad18 monoubiquitination of PCNA and allows replication past a lesion by employing low-fidelity translesion polymerases with large active sites that can accommodate bulky lesions. Rad6 is the E2 ubiquitin-conjugating enzyme that cooperates with the E3 ubiquitin ligase Rad18 to modify PCNA to initiate PRR. However, with the E3 ubiquitin ligase Bre1, Rad6 also plays a role in regulating histone H2B-K123 ubiquitination (Briggs et al., 2002; Dover et al., 2002; Ng et al., 2002; Game and Chernikova, 2009). H2B-K123 ubiquitination promotes H3-K4

and H3-K79 di- and tri-methylation by Set1 and Dot1, respectively (Briggs et al., 2001; Miller et al., 2001; Shahbazian et al., 2005; Fuchs et al., 2009; Nakanishi et al., 2009; Takahashi et al., 2009). Given the regulation by Rad6, H2B-K123 ubiquitination and H3-K4 and H3-K79 methylation may play a role in PRR (Figure 1C). To date, no histone modifications are identified to contribute specifically to the TLS branch of PRR, but the HAT Gcn5 is required for transcription of the TLS polymerase η (Kikuchi et al., 2012).

Error-free PRR requires polyubiquitination of PCNA by Ubc13-Mms2 and Rad5, which initiates a template switch to bypass the template strand lesion and copy from the newly synthesized sister chromatid. The recombination event during error-free PRR further requires the action of Rad52 epistasis group members (Boiteux and Jinks-Robertson, 2013). Thus, a post-replication template switch is mechanistically very similar to gap-induced sister chromatid recombination, and may be marked by similar or identical histone modifications. Until recently, no particular histone modifications had been attributed to error-free PRR or gap-induced SCR. However, our group has recently found that acetylation of histone H4 by the HAT Esa1 of the NuA4 complex is needed for stability of CAG repeats in a Rad5-dependent manner (**Figure 1C**). Furthermore, the HAT activity of NuA4 is required for gap-induced SCR. The most important modifications are acetylation of H4-K12 and H4-K16, known targets of Esa1 (**Figure 1B**). Additionally, H4-K16 acetylation at the CAG repeat peaks during S phase, but then disappears, presumably once repair is complete. A dynamic nature to the histone acetylation appears to be important in maintaining genomic stability, as both HAT and HDAC mutants displayed an increased frequency of CAG repeat expansions. If histone acetylation was primarily acting to disrupt higher order chromatin compaction to open the chromatin structure (Murr et al., 2006; Shogren-Knaak et al., 2006), the HDAC mutant would have rescued genomic stability by allowing constant decompaction of the chromatin. As this was not the case, the requirement for dynamic histone H4 acetylation argues for a model in which the modification is directly affecting recruitment or turnover of repair factors to facilitate PRR of gaps via SCR.

SISTER CHROMATID RECOMBINATION

Sister chromatid recombination is a homology-mediated event that contributes to both DSB repair when a sister chromatid is available as well as post-replication single strand gap repair. Chromatin modifications associated with SCR have been identified mostly within the context of a DSB, but since the physical recombination event in DSB and PRR will be similar, it is reasonable to expect that some histone modifications will affect both repair pathways. One potential example of this is H3-K56 acetylation. Not only does H3-K56 acetylation respond to replication fork damage (Wurtele et al., 2012), but it also works with Rad52 to promote SCR during repair of a DSB (Muñoz-Galván et al., 2013; **Figures 1A,B**). H3-K56 acetylation was also shown to be important in preventing CAG repeat fragility and contractions during both replication and Rad52-dependent repair events (Yang and Freudenreich, 2010; **Figure 1D**). In human cells, TIP60-dependent H4 acetylation has been shown to promote BRCA1-dependent HR (Tang et al., 2013), and depletion of the H4K16-specific HAT MOF leads to a decrease in DSB-induced HR and sister-chromatid exchanges (Li et al., 2010; Sharma et al., 2010), suggesting that H4 acetylation is important in facilitating homology-dependent recombination events between sister chromatids.

Sister chromatid cohesion is necessary for proper alignment of homologous sequences during SCR. Contributing to this process is the RSC complex, which is required to recruit cohesin to chromosomes (Baetz et al., 2004), and also to recruit the cohesin

subunits Smc1 and Scc1 to a DSB (Oum et al., 2011). These results link chromatin remodeling to cohesin loading during recombination. Lending support to a role for RSC in SCR is that *rsc2* or *rsc7* deletions confer sensitivity to MMS during G₂ but not G₁, indicating RSC is most important after synthesis when the sister chromatid would be available as a template for repair. Indeed, the *rsc7* mutant has a decrease in spontaneous sister chromatid exchange (Oum et al., 2011), and we found that both *rsc1* and *rsc2* mutants were defective in spontaneous SCR (House et al., 2014; **Table 1**). Interestingly however, only Rsc2 is able to suppress an MMS-induced increase in SCR, implicating the Rsc2 sub-complex specifically in gap-induced SCR (**Table 1**; House et al., 2014). Additionally, the Rsc2 sub-complex is detected by ChIP at an unstable CAG repeat coincident with H4K16ac, suggesting a possible recruitment mechanism for this remodeler during gap-induced repair from the sister chromatid (House et al., 2014). Both efficient γH2AX modification at a break site (Kent et al., 2007) and MRX recruitment to a DSB (Shim et al., 2007) are dependent on RSC. Therefore, this chromatin remodeling complex may be a common component of HR repair induced by either a DSB or gap that links the initial damage event to the subsequent chromatin response.

In addition to histone acetylation, both histone methylation and phosphorylation are required for proper SCR (**Figure 1B**). In yeast, the histone methyltransferase Dot1 has specificity for the H3-K79 residue and is required for DSB break repair (Game et al., 2006; Game and Chernikova, 2009). In the absence of Dot1, cells lose IR-induced Rad9 foci in G₂, suggesting a role for this modification in recruitment of Rad9 specifically when a sister chromatid is present (Toh et al., 2006; Grenon et al., 2007). Further, using a physical assay to probe for recombination intermediates and monitor unequal exchange of sister chromatids upon replication of a DSB lesion, Conde et al. (2009) found that the unphosphorylatable H2A-S129A mutant and the *dot1Δ* mutant are defective in SCR and are contributing to repair by promoting sister chromatid cohesion (Conde et al., 2009).

As the chromatin modifications and remodeling required to promote single-strand gap repair, PRR, and SCR continue to be defined, it is probable that more specific combinations of histone modifications will be revealed to be distinct from those required for DSB repair. Histone modifications could contribute to altering the chromatin environment to promote recombination, either directly by changing charge interactions between nucleosomes and the DNA, or indirectly through repair factor and chromatin remodeler recruitment. Specific histone modifications and remodelers may also contribute to the overall fidelity of repair, as illustrated by those that are needed to promote SCR as well as prevent CAG repeat instability (**Figures 1B,D**).

THE CHROMATIN RESPONSE TO STRUCTURE-FORMING DNA

Non-canonical DNA topology can lead to DNA lesions and must be resolved to prevent the loss of genomic material. Inverted repeats, some direct repeats, and homopyrimidine-homopurine runs can form stable secondary structures that impede DNA processing events, such as replication and repair, causing DNA damage and genome instability (Freudenreich, 2007; Voineagu et al., 2009a; Kim and Mirkin, 2013). Since DNA structures can

cause all of the types of damage covered above, including DSBs, stalled forks, and single-strand DNA gaps or nicks, the modifications associated with structure-forming DNA will overlap with those found at these lesions. Indeed, structure-forming CGG/CCG and CAG/CTG triplet repeats induce replication fork stalling and chromosome fragility when they reach a length of 45–70 repeats (Samadashwily et al., 1997; Freudenreich et al., 1998; Balakumaran et al., 2000; Jankowski et al., 2000; Callahan et al., 2003; Kerrest et al., 2009; Voineagu et al., 2009b; Sundararajan et al., 2010), and even short triplet repeats can interfere with nick repair (Pearson et al., 2002). DNA structures, fork stalling, and unreplicated regions of DNA have also been associated with common fragile sites (Zhang and Freudenreich, 2007; Ozeri-Galai et al., 2012). Such hard-to-replicate regions will present a particular challenge to genomic integrity and histone modifications and chromatin-associated factors will be important in maintaining stability of these regions. Additionally, the properties of the repetitive DNA can affect the chromatin structure in the region by forming very stable nucleosomes or excluding nucleosomes (see below). Furthermore, the outcome of a defective repair process can be different in a repetitive DNA sequence compared to non-repeat DNA: the built-in homology surrounding a lesion within a repeat could facilitate repair, but also lead to changes in the repeat number.

CAG repeats form stable DNA hairpins (Mirkin, 2007) and are strong nucleosome positioning elements (Wang et al., 1994; Wang and Griffith, 1995; Volle and Delaney, 2012). Long CAG repeats can activate the checkpoint response (Lahiri et al., 2004; Voineagu et al., 2009a,b; Sundararajan and Freudenreich, 2011), and expansions of the CAG repeat can occur during DNA repair if the process is inefficient (McMurray, 2010). We have found that lesions at an expanded (CAG)₁₅₅ repeat are marked by histone modifications. Both γH2AX and H4 N-terminal tail acetylation at residue K16 are enriched at an expanded CAG repeat during S phase and are required to maintain stability of a (CAG)₈₅ repeat during SCR (House et al., 2014), suggesting that these histone modifications are required for high-fidelity repair of structured DNA, potentially through direct recruitment of chromatin remodelers (such as Rsc2) or other repair factors. In human cells, knockdown of class II HDAC9 leads to an increase in CAG repeat expansion frequency (Gannon et al., 2012). However, the opposite is true for HDAC3 and HDAC5, which promote CAG repeat expansions. Though the relevant target for these HDACs is unknown, it was shown that they act within the mismatch repair pathway to protect repeat stability (Gannon et al., 2012).

Whereas CAG repeats preferentially assemble nucleosomes, CGG repeats exclude nucleosomes (Wang, 2007; Kumari and Usdin, 2009). Despite the exclusion of nucleosomes, ATR is required to prevent CGG repeat expansions (Entezam and Usdin, 2008). This suggests that ATR may be phosphorylating H2AX near the CGG repeat to initiate chromatin modifications necessary for DNA repair. Given that CGG repeats are sites of replication fork stalling and chromosomal fragility, it is not surprising that histone modifications associated with DNA damage and repair are found near these sequence elements and are important for repeat stability (Usdin, 2008; Anand et al., 2012; Kumari et al., 2012).

Activation of the checkpoint response by expanded trinucleotide repeats indicates that the structures formed at these sequences are causing damage that initiates a repair event. It is possible that distinct histone modifications are contributing to repair of structured DNA, but the particular combination of modifications that are marking such lesions are only beginning to be identified.

CHROMATIN MODIFICATIONS IMPORTANT FOR NUCLEOTIDE EXCISION REPAIR (NER)

The nucleotide excision repair pathway is responsible for removing damage that distorts the DNA helix. This type of damage includes UV-induced 6-4photoproducts (6-4PPs) and cyclobutane pyrimidine dimers (CPDs), and repair requires lesion identification and excision. After lesion removal, nucleotides are re-synthesized and the DNA ends are ligated. Chromatin structure must be altered during the NER pathway, both by remodeling and modification of histones, to allow access to the damaged DNA by the proteins participating in the NER repair pathways. Important questions relevant to the NER pathway are whether chromatin relaxation occurs before or after detection of lesions, and the role of histone modifications in chromatin changes versus repair factor recruitment. In addition, chromatin structure must be re-established at the end of the repair process. There are two NER subpathways, and the pathway choice depends on if the DNA damage occurred on a DNA strand that is being actively transcribed: transcription-coupled repair (TCR) repairs damage that occurs on the transcribed strand, whereas global genomic repair (GGR) functions to repair damage that occurs on the nontranscribed strand of active genes or in inactive regions of the genome. Once the damage is recognized and repair is initiated, the two pathways use the same set of repair factors for the downstream events. For a more detailed review of NER in chromatin, see Reed (2011).

GGR SUBPATHWAY: CHROMATIN MODIFICATIONS INVOLVED IN DAMAGE RECOGNITION AND REPAIR

The GGR subpathway repairs damage that occurs on nontranscribed DNA strands, occurring mostly from exposure to UV radiation. The initial evidence that chromatin modifications occurred during the NER process came from the finding that histones were quickly acetylated after UV irradiation (Ramanathan and Smerdon, 1986). UV irradiation triggers genome-wide histone H3 and H4 hyperacetylation in yeast (Yu et al., 2005). Indeed, acetylation of histone H3, as well as other histone modifications described below, have been shown to facilitate the GGR pathway of NER (**Figure 1E**).

HISTONE ACETYLATION

After UV irradiation, histone H3-K9 and K14 were shown by ChIP to be hyperacetylated at the repressed *MFA2* promoter in yeast (Yu et al., 2005; **Figure 1E**). This hyperacetylation was dependent on the yeast HAT Gcn5, deletion of which weakened the repair of damage at *MFA2* as assayed by *in vivo* CPD removal (Yu et al., 2005). In yeast, both Gcn5 binding and the resulting histone H3-K9 and K14 acetylation require Rad16 (Teng et al., 2008), a GGR factor with a potential function in chromatin remodeling because it is a SWI/SNF-related family member. Interestingly, the increased

Gcn5 binding and H3 acetylation were dependent on both the ATPase and RING domains of Rad16, therefore both translocation and ubiquitin ligase activities are involved (Yu et al., 2011). The resulting H3 acetylation led to a more open chromatin state, measured by restriction enzyme accessibility, which was necessary for GGR (Yu et al., 2011). Recently, another factor in addition to Rad16 has been implicated in enhancing Gcn5 binding after UV damage: the histone H2A variant H2A.Z (Yu et al., 2013). Yeast strains that are deleted for *htz1* are UV sensitive, have reduced histone acetylation, and are defective in removal of DNA damage caused by UV light (Yu et al., 2013). Altogether these studies support the conclusion that UV-induced histone acetylation promotes a more open chromatin structure that is necessary for efficient repair by the NER pathway.

A role for UV-induced histone H3-K9 acetylation during NER has also been observed in human cells. One pathway involves the transcription factor E2F1, which recruits the HAT Gcn5 to UV-damaged DNA (Guo et al., 2011). siRNA-knockdown of Gcn5 impaired recruitment of NER factors XPA and XPC to sites of damage, resulting in less efficient repair of CPDs and 6-4PPs (Guo et al., 2011). A second pathway of histone H3-K9 acetylation during NER has been linked to the function of p53, a tumor suppressor gene. Localization of the HAT p300 to sites of NER was dependent on p53, and H3-K9 acetylation after UV exposure was diminished in p53 mutants (Rubbi and Milner, 2003). Disruption of p300 caused complete NER inhibition, indicating that it is a key HAT in the GGR pathway. By monitoring micrococcal nuclease (MNase) sensitivity, p53 was found to mediate global chromatin relaxation following UV irradiation (Rubbi and Milner, 2003). Therefore, H3 acetylation by both Gcn5 and p300 together may be coordinating chromatin relaxation during NER in human cells.

HISTONE METHYLATION

Another histone modification recently connected to efficient GGR is histone methylation. Mono and di-methylation of histone H3-K79 was increased in yeast strains with mutations that render the lysines on the H4 N-terminal tail unacetylatable, and the increase in methylation correlated with the severity of UV sensitivity of the H4 K to R mutations (Evans et al., 2008). This finding, therefore, suggests that histone H4 acetylation modulates histone H3-K79 methylation levels during UV damage repair (**Figure 1E**). Histone H3-K79 is methylated by the HMT Dot1, and *dot1Δ* caused sensitivity to UV (Bostelman et al., 2007). Direct evidence for a role for Dot1 and H3-K79me in GGR was obtained by observation of defective repair of CPDs in the non-transcribed strand of *RPB2* in mutants (Tatum and Li, 2011). In contrast, Dot1 and H3-K79 were not necessary for repair in the TCR subpathway, as measured by repair of the transcribed strand of *RPB2* (Tatum and Li, 2011). Therefore, H3-K79 methylation during NER is a GGR-specific modification that may signal for recruitment of the GGR machinery to recognize damage and initiate repair. These findings contrast with a previous study that showed that a H3-K79R yeast mutant displayed almost normal NER at the expressed *RPB2* gene, though NER at the transcriptionally silent cryptic mating-type locus *HML* was impaired (Chaudhuri et al., 2009). However, this study measured NER in both strands of the different loci and did not distinguish between

the two strands, which may have therefore missed detection of the repair defect in the nontranscribed strand of *RPB2* observed by Tatum and Li (2011).

In contrast to the increased H3-K79 methylation during GGR observed in yeast, there is a global decrease in trimethylation of a different residue, H3-K9, following UV irradiation in fruit flies (Palomera-Sanchez et al., 2010; **Figure 1E**). UV irradiation increased levels of the histone H3-K9 demethylase, dKDM4B, and H3-K9 demethylation is necessary for repair of the UV lesions as repair of CPDs was impaired in flies with mutated dKDM4B (Palomera-Sanchez et al., 2010). These findings regarding the contrasting role of histone methylation at different H3 residues in the GGR pathway suggest that there may be a specific methylation pattern necessary to signal and recruit factors for repair of UV-induced DNA damage. Intriguingly, Drosophila with p53 mutations had higher levels of trimethylated H3-K9 after UV exposure (Palomera-Sanchez et al., 2010). It would be interesting to determine whether p53 mediates chromatin relaxation in flies, and whether this affects demethylase recruitment as it does HAT recruitment (see above).

HISTONE UBIQUITINATION

Histone ubiquitination has also been implicated in NER. In human fibroblasts, UV-induced DNA damage resulted in monoubiquitination of H2A-K119, but this modification was not observed in NER-deficient fibroblasts (Bergink et al., 2006; **Figure 1E**). As at DSBs, the NER-induced H2A ubiquitination was dependent on the E2-conjugating enzyme Ubc13 and the ubiquitin E3 ligase RNF8 (Marteijn et al., 2009). Additionally, the UV-damaged DNA-binding protein complex (UV-DDB) contains the subunit DDB2, a ubiquitin E3 ligase that targets histone H2A (Kapetanaki et al., 2006). Ubiquitination of H2A after exposure to UV was shown to be defective in cells from XP group E (XP-E) patients, who have a defect in UV-DDB (Kapetanaki et al., 2006). The ubiquitinated H2A may serve as a recognition signal for damage repair by NER factors that have ubiquitin-binding domains, such as RAD23B, which forms the damage recognition complex with XPC during the initial step of GGR (Kapetanaki et al., 2006). Overall, these findings highlight important associations between histone H2A ubiquitination and the NER pathway.

HISTONE PHOSPHORYLATION

A chromatin mark that is a hallmark of DSBs, γH2AX is also induced in a NER-dependent manner in UV-exposed non-replicating human cells (O'Driscoll et al., 2003; **Figure 1E**). ATR is the primary kinase for NER-dependent γH2AX (Matsumoto et al., 2007). The precise function of γH2AX in NER remains to be clarified, but if it functions similarly to its role at DSBs, it may be involved in initiating repair events necessary for recruitment of NER factors.

TCR SUBPATHWAY: POSSIBLE ROLE FOR CHROMATIN MODIFICATIONS

The TCR pathway is activated when RNA polymerase II (RNAPII) stalls at lesions, recruiting factors for repair. Thus some histone modifications associated with active transcription may also have functions in the TCR pathway.

Cockayne syndrome group B (CSB) protein and its homolog Rad26 in yeast are members of the SWI/SNF family of chromatin remodeling enzymes and are important for TCR (Guzder et al., 1996; Selby and Sancar, 1997a). A study by Fousteri et al. (2006) used a co-IP assay to identify proteins associated on the same chromatin fragment after UV treatment. Interactions between CSB, CSA (another TCR factor), and stalled RNAPII were identified, along with the HAT p300 and nucleosome binding protein HMGN1. CSB was necessary for the recruitment of the HAT p300 to stalled RNAPII, whereas the recruitment of HMGN1 was mediated via both CSB and CSA. The interaction between p300 and RNAPII was stimulated by UV and occurred prior to incision of lesions (Fousteri et al., 2006). Given the established role of p300 in NER (see above), it may be that histone acetylation is also needed to facilitate TCR.

In yeast, the association of the TCR factor Rad26 with chromatin is dependent on the presence of elongating RNAPII, and Rad26 is unable to identify lesions in the absence of transcription (Malik et al., 2010). ChIP experiments revealed that histone H3-K36 methylation stimulated the interaction of Rad26 with DNA (Malik et al., 2010). Though not yet tested, the association with Rad26 suggests that H3-K36 methylation may play a role in TCR (Figure 1E). However, since Rad26 also promotes transcriptional elongation, it may also be needed more generally to facilitate interaction of Rad26 with chromatin during transcription, rather than having any specific role during TCR.

A connection between ubiquitination and TCR was recently discovered. The deubiquitinating enzyme USP7 is brought to TCR complexes and stabilizes CSB. (Schwertman et al., 2012). TCR factors, including CSB, are known to be ubiquitinated and these could be targets of USP7 activity during TCR, potentially to protect TCR factors from UV-induced degradation. USP7 also deubiquitinates histone H2B and was recently implicated in base excision repair (BER; Khoronenkova et al., 2011). With several possible USP7 targets, the relevant ones for TCR remains to be established.

CHROMATIN REMODELING IN THE NER PATHWAY

As touched on above, chromatin accessibility plays a key role in NER, and histone acetylation and remodeling may work together to increase access to lesions for repair. Chromatin remodeling during NER has been summarized in a recent review, and compared to remodeling during repair of DSBs by the HR and NHEJ pathways (Lans et al., 2012). The role of chromatin remodelers in the NER subpathways will be highlighted here.

REMODELING IN THE GGR SUBPATHWAY

In yeast, the GGR factor Rad16 is a SWI/SNF-related family member with ATPase activity (Table 1). The ATPase activity of Rad16 is required for efficient repair (Ramsey et al., 2004; Yu et al., 2011), and it is therefore assumed that Rad16 is acting as a chromatin remodeler, although nucleosome displacement by Rad16 has not been directly observed. In addition, Rad16 has been shown to promote Gcn5-dependent histone H3 acetylation during the repair of UV damage, and this leads to increased chromatin accessibility that is necessary for efficient damage repair (Yu et al., 2011).

A link between SWI/SNF chromatin remodeling and NER was discovered in yeast and is now well established. The NER damage recognition complex consisting of Rad4 and Rad23 (yeast homolog of human XPC-RAD23B) co-purified with Snf6 and Snf5, both SWI/SNF chromatin remodeling complex subunits, and the interactions increased with UV exposure (Gong et al., 2006; Table 1). Inactivation of SWI/SNF via *snf6Δ* reduced restriction enzyme accessibility and affected the rate of CPD removal at the silent *HML* locus, implying that SWI/SNF is remodeled during NER (Gong et al., 2006). The double mutant *rad16Δ snf6Δ* was more UV sensitive than the *rad16Δ* single mutant, suggesting that Snf6 may have a role in TCR as well as GGR (Gong et al., 2006). Since Snf6 interacts with Rad4–Rad23 and Rad4 functions in both NER pathways (Verhage et al., 1994), it is possible for Snf6 to influence repair by both GGR and TCR. Additionally, following UV irradiation, chromatin was remodeled to increase DNA accessibility at *MFA2*, measured by restriction enzyme accessibility, which was partially dependent on the activity of the SWI/SNF ATPase Snf2 (Yu et al., 2005; Table 1). Overall, these findings support a function for SWI/SNF remodeling in the NER pathway.

Evidence for SWI/SNF chromatin remodeling during NER in mammals comes from BRG1 knockdown experiments that showed reduced repair of CPDs following damage with UV radiation, whereas restoring BRG1 in cells lacking the endogenous protein showed stimulation of NER (Zhang et al., 2009; Zhao et al., 2009; Table 1). In addition, SWI/SNF subunits BRG1 and SNF5 have been shown to physically interact with XPC (Ray et al., 2009; Zhao et al., 2009). In *C. elegans*, orthologs of mammalian SWI/SNF, including BRG1, BRM/SMARCA2, SNF5, PBRM1, and BAF155/SMARCC1 were implicated in survival after UV exposure (Lans et al., 2010).

A recent study revealed a function for poly (ADP-ribosyl)ation and chromatin remodeling during NER repair. Immunoprecipitation of DDB2 complexes and subsequent mass spectrometry analysis of the interacting proteins identified PARP1 as a DDB2-associated factor in human cells (Pines et al., 2012). This interaction was dependent on UV irradiation and promoted the synthesis of poly(ADP-ribose; PAR) chains in chromatin with UV-induced lesions. In DDB2-deficient cells, there was substantially less PAR immunofluorescence at UV damaged sites compared to wild-type (Pines et al., 2012). The poly(ADP-ribosyl)ation recruited the SWI/SNF chromatin remodeler ALC1 to sites of UV-induced DNA lesions. Knockdown of ALC1 using shRNA resulted in UV-sensitive cells that had deficient repair of CPDs and 6-4PPs, indicating that ALC1 activity is critical to the GGR/NER pathway (Pines et al., 2012; Table 1).

Chromatin remodeling by INO80 is also implicated in NER (Table 1). Yeast *ino80Δ* mutants and mammalian cells with RNAi knockdown of Ino80 are UV sensitive (Shen et al., 2000; Wu et al., 2007). In yeast, UV-induced recruitment of Ino80 to chromatin occurs through interactions with the Rad4–Rad23 NER damage recognition complex (Sarkar et al., 2010). In mammals, Ino80 is recruited to UV-damaged chromatin, and deletion of two INO80 complex subunits, INO80 and ARP5, resulted in defective repair of UV lesions (Jiang et al., 2010). In addition, INO80-deficient cells failed to recruit the NER factors XPC and XPA, suggesting that INO80 chromatin remodeling may be necessary for lesion

recognition and incision (Jiang et al., 2010). The links between Ino80 and NER in both yeast and mammalian systems, the UV repair defects, and the direct interactions with NER factors all support the conclusion that Ino80 is another chromatin remodeler with an important role in the NER pathway.

REMODELING IN THE TCR SUBPATHWAY

Cockayne syndrome group B and its homolog Rad26 in yeast are DNA-dependent ATPases of the SWI/SNF family of ATP-dependent chromatin remodeling enzymes acting in the TCR pathway (Guzder et al., 1996; Selby and Sancar, 1997a). Both CSB and Rad26 have been shown to affect chromatin structure, based on *in vitro* experiments for CSB, and mutant phenotypes for Rad26 (Citterio et al., 2000; Gregory and Sweder, 2001; **Table 1**). In addition, both CSB and Rad26 enhance transcriptional elongation (Selby and Sancar, 1997b; Lee et al., 2001, 2002). CSB has been shown to be necessary for recruitment of repair factors to sites of damage repaired by the TCR pathway (Fousteri et al., 2006). Recently, Rad26 was found to promote ejection of the H2A-H2B dimer during transcription of the *GAL1* gene (Malik and Bhaumik, 2012). This regulation of chromatin structure by Rad26 is critical for transcription and may be necessary for recruitment of repair factors during TCR to allow access to the DNA lesions. Future studies should directly address whether the role of Rad26 in promoting H2A-H2B dimer eviction also contributes to efficient TCR.

There are some suggestions of ISWI chromatin remodeling in NER and/or TCR. In experiments using synthetic dinucleosomes containing 6-4PPs, recombinant Drosophila ACF stimulated lesion excision (Ura et al., 2001; **Table 1**). Also, knockdown of ISWI in human cells and *C. elegans* results in a modest UV sensitive phenotype (Lan et al., 2010; Lans et al., 2010; Sanchez-Molina et al., 2011). Additionally, the human ISWI isoform SMARCA5 is recruited to UV-induced DNA lesions, where it promotes binding of the TCR factor CSB and restart of damage-stalled transcription (Aydin et al., 2014). Intriguingly, purification of the human WICH complex (WSTF-SNF2H), an ISWI family complex, identified an interaction with CSB that was confirmed by co-immunoprecipitation (Cavellan et al., 2006), adding another link between ISWI and TCR. Thus, ISWI remodeling may work together with Rad26/CSB to facilitate lesion repair during transcription.

BASE EXCISION REPAIR (BER) WITHIN CHROMATIN

DNA bases damaged by, for example, oxidation and alkylation, are repaired through BER. The damage repaired by BER does not significantly distort the DNA and therefore does not stall the replication or transcription machinery. The BER pathway is initiated when a glycosylase enzyme recognizes and excises the damaged base, leaving an abasic site. The abasic site is processed by apyrimidinic/apurinic endonuclease (APE), which cleaves the phosphodiester backbone, leaving a base gap. Then, DNA polymerase inserts the missing base(s) and DNA ligase seals the nick, completing the BER repair process. Although the role of chromatin structure in the BER pathway has not been investigated in depth, some links to histone modification and remodeling have been identified.

There is some recent evidence for the importance of histone modifications during BER. USP7 is a ubiquitin-specific human protease which deubiquitinates histone H2B *in vitro*, though it also targets non-histone substrates that include p53 (Li et al., 2002). Upon siRNA knockdown of USP7, the levels and activity of BER enzymes were not changed, but the accessibility of DNA and the repair rate of oxidative lesions were both reduced (Khoronenkova et al., 2011). These results support their model for H2B ubiquitination state affecting BER, though it will be important to address whether histone H2B, or another protein substrate, is the relevant *in vivo* target during BER. A connection between acetylation and the BER pathway was observed in mammalian cells by co-immunoprecipitation and co-localization of thymine DNA glycosylase (TDG) and the HAT p300 (Tini et al., 2002). P300/TDG complexes are competent for histone acetylation and TDG itself is also acetylated by p300, therefore TDG may be the relevant target of p300 (Tini et al., 2002). To date, no other histone modifications have been demonstrated to affect BER and therefore this is an interesting area for further study.

Multiple *in vitro* studies have investigated whether BER enzymes can function properly in the context of a nucleosome-containing template. Using uracil-containing oligonucleosome arrays, the activities of uracil DNA glycosylase (UDG), which recognizes uracil in DNA, and APE, which recognizes abasic sites, were both uninhibited, suggesting that the initial steps of BER by UDG and APE can act efficiently in intact chromatin (Nakanishi et al., 2007). However, synthesis by the polymerase functioning in BER, DNA polymerase β , was significantly reduced in the oligonucleosome array (Nakanishi et al., 2007). This inhibition was lessened upon addition of purified yeast Isw1 and Isw2, both chromatin remodelers of the ISWI family, suggesting that remodeling could be crucial for later repair events in BER within compact chromatin (Nakanishi et al., 2007; **Table 1**). The *in vitro* mechanism of BER has also been studied using an 8-oxo-7, 8-dihydroguanine (8-oxoG) lesion on reconstituted nucleosomes. Activities of murine 8-oxoguanine DNA glycosylase (OGG1), human APE, and human polymerase β were all reduced compared to their activity on a naked DNA substrate (Menoni et al., 2007). The addition of the yeast SWI/SNF complex stimulated the activity of all three BER enzymes in the repair of the oxidative lesion in the nucleosomal array, to a level comparable to their activity on naked DNA (**Table 1**). This effect required SWI2/SNF2 dependent remodeling but not relocation of nucleosomes (Menoni et al., 2007). These two *in vitro* studies utilized different types of BER lesions in the context of a nucleosome substrate. Both concluded that chromatin remodeling promotes polymerase β activity, however, differences were seen for the activities of the glycosylases (UDG, OGG1) and endonuclease APE on the nucleosome substrates that were used, which may be related to the lesion type or the nucleosome substrate itself. Overall, these studies both point to a role for the SWI/SNF family remodelers for efficient BER, though an *in vivo* role must still be established. Recently, it was shown that depletion of STH1, the ATPase subunit of RSC, results in sensitivity to MMS, and BER is considerably inhibited in cells lacking STH1 (Czaja et al., 2014). This establishes the first *in vivo* link between chromatin remodeling and BER.

HISTONE MODIFICATIONS AND NUCLEOSOME REMODELING DURING MISMATCH REPAIR (MMR)

Postreplication mismatch repair (MMR) is initiated when a base mismatch escapes the DNA polymerase proofreading machinery. In human cells, MMR is regulated by histone H3-K36 trimethylation (**Figure 1F**). H3-K36me3 is required *in vivo* to recruit the heterodimer MSH2-MSH6 (MutS α) to chromatin through the Pro-Trp-Trp-Pro (PWWP) domain of MSH6, a domain that specifically interacts with H3-K36me3 (Li et al., 2013). Since H3-K36me3 is abundant during G1 and early S phase, it is thought that this ensures the enrichment of MutS α on DNA during the period when mismatches are likely to arise. Cells that lack the H3-K36 trimethyltransferase SETD2 have altered MSH6 localization and a mutator phenotype, but are not defective in MMR *in vitro* (Li et al., 2013). Whether additional histone modifications are involved in MMR is as yet unknown; good candidates may be those associated with the progression of DNA replication, such as H3-K56 (Kadyrova et al., 2013).

Because nucleosomes become disassembled in front of a replication fork, newly replicated DNA is relatively nucleosome-poor and MMR may not need robust chromatin remodeling to effectively compete with nucleosomes. However, fully formed nucleosomes have been observed about 250 bp from a replication fork, and there are intermediates in the assembly process in the region in between (Sogo et al., 1986; Jackson, 1988). Therefore, the MMR machinery is likely to encounter some completely formed nucleosomes in addition to nucleosome intermediates. There is some evidence for interaction between MMR factors and the histone H3–H4 chaperone chromatin assembly factor 1 (CAF-1). Mismatch correction reactions with HeLa cell extracts demonstrated that replication error correction occurs on DNA that is packaged into nucleosomes by CAF-1 (Kadyrova et al., 2011). However, in a combined *in vitro* MMR and nucleosome assembly assay, a mismatch in a nicked plasmid substrate delayed loading of nucleosomes in a human cell extract (Schöpf et al., 2012), suggesting that MMR interferes with nucleosome assembly. The balance between MMR and chromatin reassembly may be regulated by a physical interaction between MutS α , specifically MSH6, and the p150 subunit of CAF-1 (Schöpf et al., 2012).

In addition to these interactions between MMR and chromatin assembly factors, passive chromatin remodeling assists the MMR process. Using *in vitro* experiments with reconstituted nucleosomes and purified human proteins, the MMR initiation heterodimer MutS α disassembles nucleosomes (Javaid et al., 2009). Nucleosome remodeling by MutS α required a mismatch and translocation of the complex as a sliding clamp along the DNA (Javaid et al., 2009). The nucleosome remodeling function required ATP binding but not hydrolysis, suggesting that the remodeling function is passive. Histone H3 acetylation or an H3 acetylation mimic (H3-K115Ac, H3-K122Ac, H3-K56Q), enhanced the remodeling function of MutS α (Javaid et al., 2009). Additionally, phosphorylation of histone H3-T118 enhanced nucleosome disassembly by MutS α by 25-fold *in vitro* (North et al., 2011). However, no *in vivo* investigation has been done yet to support a role for H3 phosphorylation or acetylation in MMR. There is also evidence that passive MutS α -dependent nucleosome

disassembly may not be sufficient, as human MutS α bound poorly to a substrate with a mismatch within a nucleosome (Li et al., 2009). In addition, nucleosomes blocked ATP-induced sliding of MutS α along the DNA when there was a mismatch between two nucleosomes (Li et al., 2009). Overall, these findings indicate that nucleosomes likely inhibit the MMR process to some degree, and active remodeling may yet be found to play a role in MMR.

CONCLUDING REMARKS

The interplay between histone modifications and DNA repair likely creates a diverse array of cellular responses to DNA damage based on the type of lesion and the preferred pathway of repair for a particular lesion. γ H2AX is the first detectable histone modification in response to DSBs, but it appears to be a general initial modification, acting as a broad signal of DNA damage, activating signaling cascades in response to stalled forks, gaps, DNA structures, and UV lesions, as well as DSBs. The subsequent, downstream histone modifications may guide repair to the appropriate pathway based on lesion type.

A major unanswered question for many of the histone modifications summarized here is their mechanism of action. Do histone marks recruit specific repair factors or remodelers via direct interaction, or change local chromatin accessibility in a more general way, or a combination of both? Several examples of direct interactions exist, for example Arp4, a subunit of INO80, SWR1, and NuA4 complexes, binds specifically to yeast H2A phosphorylated at Ser129 (Downs et al., 2004). In mammalian cells, the repair mediator MDC1 binds directly to γ H2AX via tandem BRCT domains (Uziel et al., 2003; Lukas et al., 2004; Stucki et al., 2005). In addition, other roles for modifications can also be envisioned, such as repositioning of the damaged area to another nuclear compartment to direct appropriate repair (Dion and Gasser, 2013).

Specific combinations of histone modifications may also be important to differentially favor the recruitment of particular repair factors. Depending on the interaction of the repair proteins with the histone modifications, progressive histone modifications after DNA damage could influence repair pathway choice or progression. One example of this during DSB repair in human cells is Tyr142 on H2AX, which is phosphorylated in the absence of DNA damage by the WSTF kinase (Xiao et al., 2009). However, upon DNA damage and phosphorylation of H2AX at Ser139, Tyr142 is dephosphorylated by the Eya1 and Eya3 tyrosine phosphatases (Cook et al., 2009). While the di-phospho γ H2AX can be bound by the repair factor MCPH1, MDC1 only efficiently binds γ H2AX once it is dephosphorylated at Tyr142 (Singh et al., 2012), thus directing binding of repair factors in an orderly fashion. In most cases, relatively little is understood about the order of the occurrence of the modifications depicted in **Figure 1**, and whether some work together to recruit factors, change chromatin structure, or signal completion of repair.

It is reasonable to expect that different lesions will also require a different chromatin environment to promote repair, and thus unique levels and types of chromatin remodeling. End resection, D-loop extension during HR, gap filling, fork restart, and repair of base lesions or mismatches could each require a certain degree of nucleosome movement or displacement. For instance,

if a homologous template is utilized for repair, chromatin remodeling will require the movement of several nucleosomes at the targeted, homologous sequence to allow invasion into the template sequence and subsequent copying, as well as at the site of the lesion if any resection is required for repair; on the other hand, repair of base lesions or gap filling without strand invasion may not require as substantial of a remodeling process. Finally, repair resolution will require reestablishment of the chromatin state and DNA damage checkpoint recovery. Depending on the chromatin modifications that took place during repair, the disruption to the chromatin will vary and thus may require different factors to reestablish the normal chromatin state.

Some combinations of histone modifications that distinguish repair pathways from one another are summarized here, but many remain to be identified. Understanding how these histone modifications work together to contribute to repair will further our understanding of how the DNA repair machinery functions within the context of the chromatin structure. Additionally, roles for chromatin modifications in designating repair choice, orderly progression of repair, turnover of repair factors, and resolution of the damage response may be revealed.

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Do chromatin changes around a nascent double strand DNA break spread spherically into linearly non-adjacent chromatin?

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In the last decade, a lot has been done in elucidating the sequence of events that occur at the nascent double strand DNA break. Nevertheless, the overall structure formed by the DNA damage response (DDR) factors around the break site, the repair focus, remains poorly understood. Although most of the data presented so far only address events that occur in chromatin *in cis* around the break, there are strong indications that in mammalian systems it may also occur *in trans*, analogous to the recent findings showing this if budding yeast. There have been attempts to address the issue but the final proof is still missing due to lack of a proper experimental system. If found to be true, the spatial distribution of DDR factors would have a major impact on the neighboring chromatin both *in cis* and *in trans*, significantly affecting local chromatin function; gene transcription and potentially other functions.

Keywords: DNA damage, double strand DNA break, chromatin, DNA damage response, gamma-H2AX, ATM, RNF168

INTRODUCTION

Double strand DNA breaks (DSBs) are one of the most dangerous genetic lesions the DNA can incur. They can occur exogenously or endogenously; they are mostly random but can also be programmed as a part of a wider biological process (mating type switching in yeast, meiotic recombination, V(D)J recombination and class-switch recombination in mammals). They can be repaired through two complementary pathways: the faster but potentially error-prone non-homologous end joining (NHEJ) and through slower and more precise homologous recombination (HR). These pathways are to a large extent conserved from yeast to humans, but the repair pathway of choice can vary between species – from predominantly HR-mediated repair in yeast to predominantly NHEJ in humans. Notably, if left unattended, such DSBs can induce cell death while their improper repair can result in alterations of the genetic makeup of the cell, in higher organisms potentially leading to oncogenic transformation.

Double strand DNA breaks induction and subsequent repair does not occur in isolation, but in the context of a nucleoprotein superstructure called chromatin. Chromatin is a highly repetitive structure based on the 146 bp of DNA wound around a histone octamer, together termed a nucleosome, and connected via linker DNA of varying length. This creates a bead-on-a-string structure which can be compacted further, resulting in overall DNA compaction of up to a 100,000-fold in order to fit into the nucleus. Such high compaction necessitates an organized structure within the DNA to allow for proper function and prevent entanglement. There are varying levels of compaction of specific genomic regions (reviewed in Gilbert et al., 2005), depending on the association of additional proteins to histones.

Structural differences in chromatin compaction frequently reflect the function of various chromatin regions. Less compacted euchromatin regions tend to be more transcriptionally active and the more compacted heterochromatin ones predominantly silent. Moreover, heterochromatin tends to cluster in the center of a chromosome territory, while euchromatin forms the periphery and the interchromosomal interaction surfaces (reviewed in Gilbert et al., 2005). This creates a significant potential for physical proximity between linearly distant intrachromosomal regions, or between regions on different chromosomes. Mostly it's passive proximity through folding and compaction, but some may be both active and targeted, like the concerted interaction of genes from different chromosomes: for better response to signaling (Spilianakis et al., 2005), for regulated (Lomvardas et al., 2006) or concerted transcription (Lin et al., 2009).

Complex chromatin structure creates a significant challenge in both recognition and repair of DSBs. A nascent break needs to be sensed and the repair machinery needs to have access to the broken ends. Most DSBs are sensed and repaired very fast via NHEJ, but a fraction of them persists and requires the activation of the full DNA damage response (DDR) signaling. This assists the repair mechanisms in preventing separation of broken ends (Bredemeyer et al., 2008), helps avoid promiscuous repair and prevents DNA replication or mitosis to initiate prior to physical repair (reviewed in Ciccia and Elledge, 2010).

ATM ACTIVATION AT THE ONSET OF DDR SIGNALING

One of the earliest events in the activation of DDR signaling is activation of the ataxia telangiectasia-mutated (ATM) kinase. When inactive, ATM exists as a homodimer that dissociates upon activation, creating two enzymatically active monomers

(Bakkenist and Kastan, 2003). Active ATM can phosphorylate numerous targets, both located proximally to the break site and inducing focal accumulation of DDR factors (together termed an IRIF – ionizing radiation-induced focus) or dispersed throughout the nucleus (Matsuoka et al., 2007). Activation is concomitant with phosphorylation of the monomer at several sites, including S1981 (subsequently termed pATM). The phosphorylation occurs through cross-phosphorylation within the ATM dimer, and in the case of humans it is required for dimer dissociation and activation (Bakkenist and Kastan, 2003). The implication is that individual inactive dimers are independently activated and that the phosphorylation is not exogenous, as previously hypothesized (Kitagawa and Kastan, 2005).

What then activates ATM and where in the nucleus does it occur? The evidence suggests that it could be through the interaction with the deprotected DNA at the site of a DSB. ATM is activated by nascent DSBs, and ChIP analyses have shown that pATM accumulates in the proximity of a break site (Berkovich et al., 2007). One of the earliest events around the break site in human cells is probably nucleosome removal and creation of a longer stretch of naked DNA, analogous to yeast (Tsukuda et al., 2005). Notably, naked DNA in excess of 200 bp is enough to activate ATM both *in vitro* and in *Xenopus* extracts (You et al., 2007), even in the absence of deprotected DNA ends. The MRN complex (Mre11/Rad50/NBS1), one of the earliest DSB sensors which binds directly to the break site (reviewed in Stracker and Petrini, 2011), binds also ATM and is required for its full activation, potentially through facilitating the interaction of ATM with the DNA. In contrast, globally chromatin relaxation through trichostatin A (TSA) or chloroquine treatment, activates ATM throughout the nucleus without forming foci (Bakkenist and Kastan, 2003). Thus, it is the localized chromatin relaxation around a nascent DSB what induces the site-specific ATM activation.

Upon activation, ATM phosphorylates numerous targets, including a histone H2A variant H2AX (Burma et al., 2001). H2AX is highly abundant in the cells and comprises 5–25% of the total nuclear H2A (Rogakou et al., 1998, 1999). It differs from the canonical H2A in having an extended C-terminus where it becomes phosphorylated on serine 139 during DDR, forming γH2AX. This initiates very early upon break induction, within seconds, and at equilibrium γH2AX region can extend up to 500 kb linearly away from the break site (Meier et al., 2007; Savic et al., 2009). γH2AX serves as the earliest histone mark which specifies the region in chromatin where a DNA break occurred. In its absence, downstream events like MDC1 (mediator of DNA damage checkpoint 1) binding, RNF168 accumulation or 53BP1 foci formation do not occur properly. This results in DNA damaging sensitivity and illustrates the importance of local chromatin in the proper repair of DNA breaks. Notably, not only cells lacking γH2AX (H2AX S139A) show DNA damage hypersensitivity, but the mutants overexpressing phosphomimetic S139E as well (Celeste et al., 2003a,b), even though H2AX S139E can constitutively activate DDR signaling (Kobayashi et al., 2009). This suggests that it is the absence of local accumulation what is impairing DNA repair in H2AX S139E, and not any reduction in global DDR signaling.

MAKING THE CASE FOR THE FORMATION OF γH2AX and K63-UBIQUITYLATION IN 3D

γH2AX recruits MDC1 which in turn binds activated ATM and retains it near the break site (Stucki and Jackson, 2006; Berkovich et al., 2007; So et al., 2009). This has led to the hypothesis that the γH2AX-dependent recruitment of MDC1 and pATM creates a feed-forward mechanism that leads to an extended γH2AX region (Stucki and Jackson, 2006). Subsequent results have confirmed that in the absence of ATM γH2AX levels are reduced in both extent and density (Savic et al., 2009). However, the absence of MDC1 had no effect on the extent of the γH2AX-containing region even though it reduced the peak intensity to the ATM^{-/-} levels, indicating that only the high, proximal γH2AX levels are dependent on MDC1 anchoring pATM on chromatin and that the distal γH2AX is independent of this mechanism (Savic et al., 2009).

The question that arises is how is this distal γH2AX then formed? As mentioned, ATM activation is probably site specific and it occurs at the DSB, but many of the ATM targets do not localize to the break site. Thus, a fraction of the activated ATM has to diffuse from the break site and phosphorylate targets throughout the nucleus. The resulting concentration gradient of active pATM molecules could be the defining factor in determining the γH2AX spread. Distribution of pATM upon laser stripe-mediated DNA damage induction indicates an initial pATM accumulation at the stripe subsequently followed by the overall increase in the pATM signal throughout the nucleus, fitting with the idea of localized activation followed by diffusion (Kruhlak et al., 2006). Notably, when chromatin is globally induced to relax through TSA or chloroquine treatment, ATM is activated globally, without forming foci (Bakkenist and Kastan, 2003). Thus, the site-specific changes in chromatin around nascent DSBs are what induces site-specific ATM activation.

Diffusible pATM as the generator of distal γH2AX would indicate that such a chromatin mark can be deposited non-linearly and does not require tracking along the DNA fiber (**Figure 1A**). In fact, γH2AX formation in human cells at unprotected telomeres can form discontinuously (Meier et al., 2007). Although the mechanisms are somewhat different, it is also of note that in budding yeast the H2A phosphorylation equivalent to γH2AX (γH2A) can skip over heterochromatic regions (Kim et al., 2007), supporting the idea that the γH2AX spreading may not occur through chromatin tracking.

pATM diffusion hypothesis suggests that at high enough concentration, pATM may even phosphorylate H2AX and generate the DDR cascade independently of the break site, analogous to the skipping of chromatin regions (**Figure 1A**). Notably, artificially high concentrations of NBS1 or Mre11 can lead to activation of the downstream DDR cascade and formation of an IRIF even on undamaged chromatin (Soutoglou and Misteli, 2008). The reason may be that Mre11 and NBS1 can bind and recruit non-phosphorylated ATM to damaged chromatin (So et al., 2009), thus bringing ATM in close proximity to the DNA which may be enough to activate it (You et al., 2007). The artificial accumulation of ATM itself was enough to activate the initial damage response and γH2AX formation, but curiously did not elicit a more downstream 53BP1 accumulation (Stewart et al., 2003). The intriguing

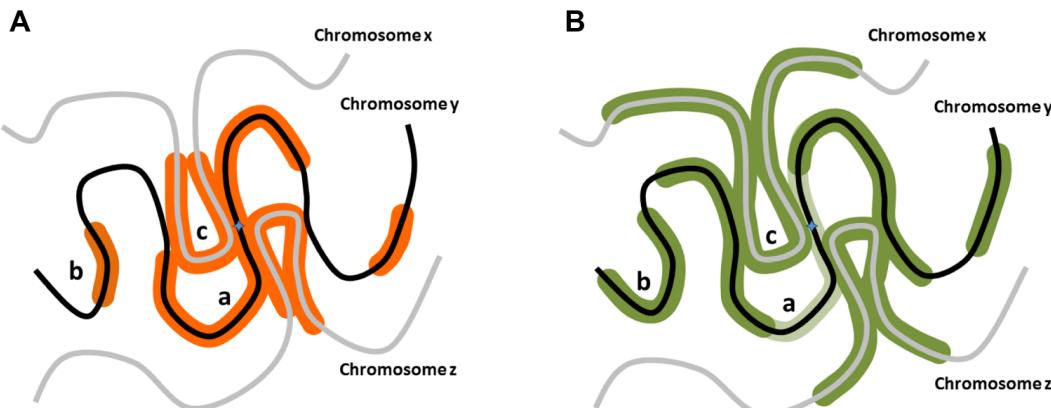


FIGURE 1 | Potential distribution of DNA damage associated modifications around nascent DSBs. (A) When a DSB occurs on a chromosome y, aside from the confirmed linear phosphorylation in the vicinity of the break site along the chromosome y (a), H2AX could be phosphorylated on distal chromosomal regions of the same chromosome (b), or on regions of different chromosomes (chromosomes x, z) in the vicinity of the break site (c).

(B) RNF168 polyubiquitylation-dependent 53BP1 distribution could exhibit distribution analogous to γ H2AX, but potentially more expanded distally from the break site. Notably, in G2 stage of the cell cycle in particular, 53BP1 distribution pattern may be only partially overlapping with the γ H2AX region as it is excluded from the vicinity of the break site bound by BRCA1 (light green; Chapman et al., 2012).

possibility is that chromatin anchoring of ATM (by means of LacO/LacI-ATM association) is not enough to trigger 53BP1 focus formation, but the endogenous ATM, initially recruited and activated by chromatin-associated MRN and subsequently diffusing away from the break, is what elicits 53BP1 foci.

RNF168, ubiquitin ligase functioning downstream of ATM in DDR, displays properties similar to the ATM in the γ H2AX spatial distribution hypothesis. It is a ubiquitin ligase that creates lysine 63-linked polyubiquitin chains focally around the DSBs in chromatin, among others on histone H2A (Doil et al., 2009; Stewart et al., 2009; Panier et al., 2012). This is dependent on the preceding H2A monoubiquitylation via RNF8, which in turn is dependent on MDC1 and serves as an anchor and a primer for the polyubiquitin assembly (Doil et al., 2009; Stewart et al., 2009). Although true for initial recruitment and activity, subsequent ubiquitylation of chromatin seems independent of RNF8, thus it appears that RNF168 has an autoregulatory effect on its own chromatin recruitment and signal amplification capacity. This would indicate that the major way RNF168 is regulated at break sites is through overall availability, which is exactly what a recent study showed (Gudjonsson et al., 2012). There, the size of individual 53BP1 foci formed around a site-specific breaks was increased in the cells lacking TRIP12 and UBR5, the two E3 ubiquitin ligases which regulate RNF168 turnover. Moreover, the incremental increase in the ionizing radiation gradually reduced the size of 53BP1 foci in TRIP12^{siRNA}/UBR5^{siRNA} cells, but the foci were nonetheless larger than in equivalent controls, suggesting RNF168 enzyme availability is a factor regulating 53BP1 focus size.

The proposed mechanism through which RNF168 could lead to spatial ubiquitylation and 53BP1 recruitment is somewhat similar to the way γ H2AX is induced by ATM activity (Figure 1B). RNF168 association with chromatin requires chromatin to be primed through RNF8-mediated ubiquitylation of histone H2A, thus creating the binding sites for RNF168 (Panier et al., 2012). Similarly, ATM recruitment to chromatin requires prior priming

through γ H2AX induction which creates MDC1 binding sites (Stucki and Jackson, 2006). In contrast to ATM, which can serve as its own priming enzyme, RNF8 is essential for RNF168 function at break sites (Doil et al., 2009; Stewart et al., 2009). Subsequent to initial binding, RNF168 may extend the monoubiquitin tag of the binding site but may also be able to polyubiquitylate the neighboring nucleosomes and create new binding sites irrespective of RNF8 (Panier et al., 2012). Crucially, the RNF168-mediated ubiquitylation has not been shown to have any DNA tracking ability, thus it may depend on the proximity of the substrate nucleosomes. This creates a potential for a feed-forward mechanism and signal to jump between chromatin regions, if the latter is looped close to the original source of signaling. The ability of 53BP1 foci to grow significantly larger than to γ H2AX-containing seeding region, proportionally to the amount of available RNF168 in the cell (Gudjonsson et al., 2012), strongly supports the idea of RNF168-mediated ubiquitylation spreads beyond the confines of γ H2AX-coated chromatin region (Figure 1B).

Chapman et al. (2012) go even further and show that 53BP1 foci preferentially form outside their γ H2AX seeding regions. This is crucial structural evidence which shows that the predominant way RNF168 induces 53BP1 binding is not through inducing ubiquitylation within the region where RNF8 creates the seeding monoubiquitylation, but in the region that results from positive autoregulatory signals beyond it. Moreover, they report non-random changes in the structure of such 53BP1 foci, depending on the cell cycle stage where the DSBs are induced and on the central presence of BRCA1. This indicates that IRIF formation, at least at the level of 53BP1 binding, may indeed be three dimensional, forming a regulatable sphere around a putative γ H2AX or BRCA1-demarcated break site. Unfortunately, due to the constraints of the immunofluorescent analysis, it is impossible to say whether the resultant globular structure includes 53BP1 coating non-linear regions of chromatin or is the result of a specific folding structure of the linearly adjacent chromatin loops.

THE CONUNDRUM

There was at least one attempt to answer the question of spatial effect a DSB on chromatin (Iacoboni et al., 2010). The ChIP-Seq analysis in a human cell line showed no apparent spatial γ H2AX spread, since there was no detectable γ H2AX accumulation outside of the linear chromatin regions harboring target sequences for enzymatically induced DSB. Unfortunately, this conclusion is based on a premise that chromatin is static and that in every cell in the analyzed population the same distal, inter- or intrachromosomal regions will fold back to be in close proximity to the break site. In contrast, there is a large body of evidence which shows that the position and interaction of chromatin regions is stochastic and in most cases with only moderate tendencies of association with a specific partner on a population level (reviewed in Gilbert et al., 2005), far below the requirements to support the starting premise above. The nuclear position of a genomic locus is also not fixed and a the locus can move within a confined space over time (reviewed in Dion and Gasser, 2013), potentially leading to stochastic interactions with neighboring chromatin. Moreover, the presence of a DSB seems to increase the average mobility of neighboring chromatin in mammals (Krawczyk et al., 2012), further reducing the chance of interchromosomal or interregional chromatin interactions being uniform throughout the population, implied in the conclusions by Iacoboni et al. (2010). On the other hand, if the spatial distribution hypothesis is valid, the resulting γ H2AX phosphorylation would spread stochastically throughout the genome, in accordance with the likelihood of association with different chromatin regions of the break site. In this case, the result would be a change in the ChIP-Seq signal far below the measurable level, fitting with the primary data.

Given the problem of stochastic interactions, the only way to practically address this issue is to use a cellular system where the association of the break site and a distal chromatin region is inducible and non-random. This creates a problem, as only a handful of specific interchromosomal interactions have been described in mammalian cells, and none include a site specific, inducible DSB. However, two recent reports using system of site-specific break coupled with a defined homologous donor site shed a new light and show that γ H2A can exhibit discontinuous, both intra- and interchromosomal distribution (Li et al., 2012; Renkawitz et al., 2013). In one report, Li et al. (2012) show that upon HO endonuclease-mediated break induction and activation of the HR repair mechanism, RAD51 can interact not only with the broken DNA fragment but also the homologous donor sequence on a separate chromosome as well. In the second report, Renkawitz et al. (2013) show a comparable result with the donor distally on the same chromosome. Furthermore, they showed that direct physical association during strand invasion in HR is not necessary for *in trans* γ H2A induction. In yeast, chromosome centromeres tend to be clustered and in close proximity, and they have shown that upon DSB induction in the centromeric region of chromosome IV, centromeres on chromosomes XI and XVI also become positive for γ H2A and RAD51, even in the absence of a homology donor. Crucially, these signals are not detectable if the DSB is induced more distally and not in the centromeric region. Thus, in yeast γ H2A phosphorylation can spread *in trans* to unbroken

DNA in close proximity and may not require direct physical interaction.

The Jentsch group has previously reported starkly different Rad51 findings using a similar system of a site-specific DSB, but in cells without a specific donor sequence (Kalocsay et al., 2009). Here they showed increased Rad51 signal to be exclusively intrachromosomal, non-specific and to varying degrees along the whole chromosome. However, as this system lacks a homology donor and no productive strand invasion can take place, Rad51 distribution is most probably the just a result of random homology searches. Generally, the constraints on chromatin movement result in intrachromosomal interactions being much more prevalent than interchromosomal (Lieberman-Aiden et al., 2009). In mouse lymphocytes similar constraints on broken DNA ends determined the choice of translocation partners to such an extent that intrachromosomal translocations per megabase are at least an order of magnitude more frequent than the ones across chromosomes (Zhang et al., 2012). In the case of Kalocsay et al. (2009), the same preferences probably masked the low level cross-chromosome interactions in favor of the more prominent intrachromosomal ones. The stark difference between the two reports from the same group clearly showcases how essential a targeted system is in properly addressing the *in trans* effects around a DSB and why a targeted system is critical to properly address this question in mammals.

THE EFFECTS OF 3D SPREAD

What would be the consequence of a three dimensional IRIF? Two recent studies have shown that a nascent DSB induces transcriptional silencing of a gene *in cis* (Shanbhag et al., 2010; Pankotai et al., 2012). Moreover, Shanbhag et al. (2010) have shown that this repression does not depend on the physical presence of a break, as ATM inhibition upon DSB induction abolishes transcriptional repression. It does, however, depend on the downstream DDR signaling, including γ H2AX induction and in particular subsequent RNF168 mediated ubiquitylation. If γ H2AX and polyubiquitylation through RNF168 can indeed extend beyond the linear DNA, aside from resulting in the described transcriptional inhibition could occur *in trans* as well as *in cis*. This would have a major impact on chromatin function – not only would it result in a spatial transcriptional silencing, fitting with the described overlap of 53BP1 foci and zones of reduced mRNA synthesis (Gudjonsson et al., 2012), but the changes could be even more profound and affect other chromatin functions as well.

CONCLUSION

Even though in the last decade our knowledge about the interaction networks governing the DDR in mammals has grown exponentially, the spatial organization of this network, in particular the spatial organization of an IRIF is still a mystery. Initial attempts have shown great promise and indicate some form of spatial regulation of the DDR, but the question whether DDR factors indeed accumulate in a non-linear fashion is still left unanswered. The implications of such an accumulation on the structure and function of the adjacent chromatin are many, but addressing them directly and unequivocally will have to await the development of specific, targeted approach.

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Role of p97/VCP (Cdc48) in genome stability

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Ubiquitin-dependent molecular chaperone p97, also known as valosin-containing protein (VCP) or Cdc48, is an AAA ATPase involved in protein turnover and degradation. p97 converts its own ATPase hydrolysis into remodeling activity on a myriad of ubiquitinated substrates from different cellular locations and pathways. In this way, p97 mediates extraction of targeted protein from cellular compartments or protein complexes. p97-dependent protein extraction from various cellular environments maintains cellular protein homeostasis. In recent years, p97-dependent protein extraction from chromatin has emerged as an essential evolutionarily conserved process for maintaining genome stability. Inactivation of p97 segregase activity leads to accumulation of ubiquitinated substrates on chromatin, consequently leading to protein-induced chromatin stress (PICHROS). PICHROS directly and negatively affects multiple DNA metabolic processes, including replication, damage responses, mitosis, and transcription, leading to genotoxic stress and genome instability. By summarizing and critically evaluating recent data on p97 function in various chromatin-associated protein degradation processes, we propose establishing p97 as a genome caretaker.

Keywords: p97/VCP, Cdc48, genome stability, DNA damage response, DNA replication, DNA repair, chromatin-associated protein degradation, protein-induced chromatin stress

INTRODUCTION

Genome stability is a prerequisite for cell survival, cancer prevention, and control of aging (Papamichos-Chronakis and Peterson, 2013). The genome is constantly attacked by various reactive oxygen species from endogenous sources. In addition to endogenous sources, DNA lesions can also be generated by a variety of exogenous sources, such as ionizing radiations (IR), the ultraviolet light (UV), and many chemical agents, some of which are products of our industrialized society. It is estimated that the mammalian genome accumulates thousands of DNA lesions every day that disturb DNA synthesis and cell division, two essential processes in genome amplification, preservation and transition to the next generation (Jackson and Bartek, 2009). Failure to maintain genome stability leads to chromosomal aberrations, gene mutations, and cancer or cell death. To maintain genomic stability, cells respond to DNA damage by activating a spatiotemporal signaling pathway known as the DNA damage response (DDR; Ciccia and Elledge, 2010). Following DNA damage, the DDR drives cell cycle checkpoints and initiates DNA repair or induces apoptosis if the genome is severely damaged. Although DDR after double-strand break (DSB) has been widely investigated and is considered as a DDR model, other types of DNA lesions can cause activation of lesion-specific DDRs that signal and recruit appropriate sensor, transductor, and effector proteins (Jackson and Bartek, 2009). For example, unrepaired DNA lesions that enter S phase encounter a serious problem during DNA replication. When the DNA replication fork approaches these lesions, the cell activates the DNA damage tolerance (DDT) pathway, which enables survival by activating the translesion DNA synthesis (TLS)

pathway (Friedberg, 2005). The TLS pathway enables recruitment of translesion DNA polymerases (DNA pols), which can bypass bulky DNA lesions and ensure continuous DNA synthesis. Activation of lesion-specific DDR and appropriate DNA repair mechanisms or DDT ensures cell survival and prevents genomic instability and cancer. DDR and DDT are controlled by various post-translational modifications (PTMs) in which ubiquitination and sumoylation play an essential role (Bergink and Jentsch, 2009; Al-Hakim et al., 2010; Ulrich and Walden, 2010; Bekker-Jensen and Mailand, 2011; Lehmann, 2011b; Psakhye and Jentsch, 2012). Ubiquitination and sumoylation regulate timely assembly and disassembly of various DNA repair and genome caretaker molecules. Disturbances in this tight regulation could cause severe defects in the DDR and lead to genomic instability, which has been demonstrated in certain types of breast and ovarian cancers and patients with RIDDLE syndrome (Blundred and Stewart, 2011; Lipkowitz and Weissman, 2011). Mutations in BRCA1, an E3 ubiquitin ligase involved in DDR, can result in breast and ovarian cancers, while mutations in the DDR-related E3 ligase RNFL168 can cause RIDDLE syndrome, which is characterized by radiation hypersensitivity, immunodeficiency, dysmorphic features, and learning difficulties. Because many chromatin-associated DDR proteins are tightly bound to sites of DNA damage and are often protected from degradation, spatiotemporal turnover and degradation are facilitated by the ubiquitin-proteasome system (UPS; Dianov, 2011; Levy-Barda et al., 2011; Ramadan and Meerang, 2011). It is still not known how the UPS and its largest component, the proteasome, approach, remove and degrade tightly bound protein complexes on chromatin. Discovery of a p97 function related to

chromatin may provide the answer (Dantuma and Hoppe, 2012; Ramadan, 2012).

In recent years, ubiquitin-dependent molecular chaperone p97 has emerged as an essential regulator of UPS-dependent protein turnover in chromatin. p97, also known as valosin-containing protein (VCP) or Cdc48, a central element of the UPS and is integrated downstream of substrate ubiquitination and upstream of the proteasome. With intrinsic ATPase activity, p97 extracts (segregates) polyubiquitinated proteins from diverse cellular locations and presents them for proteasomal degradation. p97 is also involved in degradation of highly folded ubiquitinated soluble proteasome substrates and thus functions as unfoldase (Beskow et al., 2009). Recently elucidated functions of p97 in several DNA metabolic processes indicate that the protein is a constitutive component of various essential chromatin-related processes in the cell cycle, DNA replication and repair, mitosis, and transcription. However, the function of p97 as it relates to genome stability has not yet been esteemed.

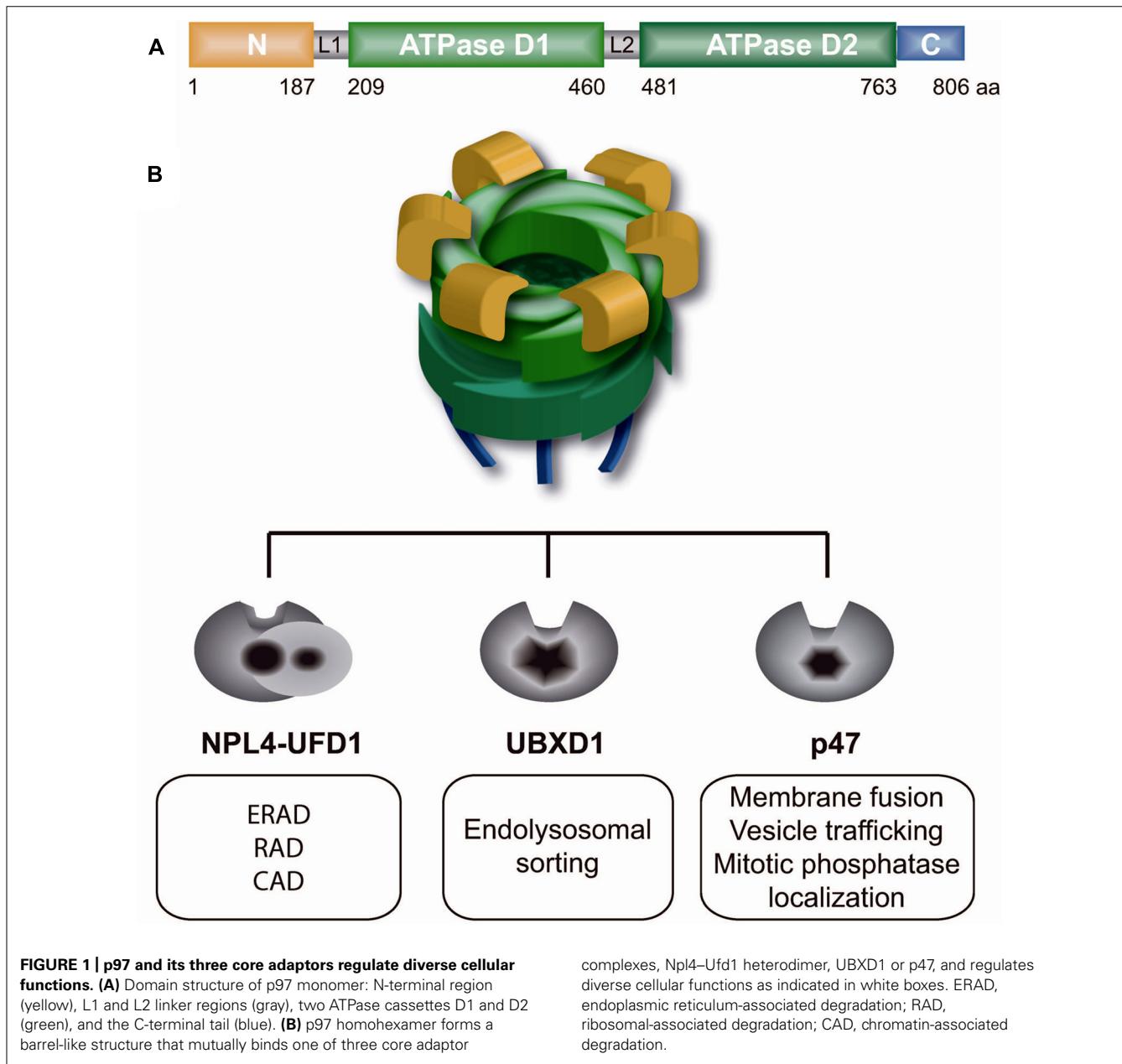
In this review, we discuss various p97 functions in chromatin-related processes essential for maintenance of genome stability and attempt to establish p97 as a genome caretaker. We also present a large body of evidence suggesting that protein-induced chromatin stress (PICHROS) induces genotoxic stress, ultimately leading to genome instability.

UBIQUITIN-DEPENDENT MOLECULAR CHAPERONE p97

Molecular chaperone p97, also known as VCP in vertebrates, Cdc48 in *S. cerevisiae*, Cdc-48 in *C. elegans*, TER94 in *Drosophila*, and VAT in archaeabacteria, is a class II member of the ATPase associated with diverse cellular activities (AAA) ATPases (Woodman, 2003; Halawani and Latterich, 2006; Jentsch and Rumpf, 2007; Stoltz et al., 2011; Meyer et al., 2012; Verma et al., 2013). p97 is highly conserved from archaeabacteria to humans and is one of the most abundant proteins in the cytoplasm and nucleoplasm (Peters et al., 1990). It is a homohexameric barrel-like molecular machine composed of the following domains: N-terminal, two ATPase cassettes (D1 and D2) and a C-terminal domain (**Figure 1A**). The N-terminal region of p97 binds a myriad of substrates primarily embedded in different cellular structures, such as the endoplasmic reticulum (ER), ribosomes, membrane vesicles, spindles, or chromosomes. After it is bound to the substrate, p97 uses ATP hydrolysis in the D2 domain to stimulate major intrinsic conformational changes that are transmitted throughout the entire p97 molecule to the N-terminal domain (Rouiller et al., 2002; Li et al., 2012). Intrinsic conformational changes allow p97 to remodel and thus extract (segregase activity) bound substrates from diverse cellular structures or protein complexes. Beside its segregase activity from different cellular structures, p97 serves as unfoldase in processing of highly folded soluble proteasome substrates (Beskow et al., 2009). The majority of p97 substrates are ubiquitinated, and ubiquitin serves as a major regulator of p97 function (Ye, 2006). Identification of *Ufd1*, one of the core p97 adapters, and *Ufd2*, a p97-associated E4-ubiquitin ligase, as essential yeast genes in degradation of soluble substrate by ubiquitin fusion degradation (UFD) pathway was the first report of p97/Cdc48 function in the UPS (Johnson et al., 1995). p97 acts as a segregase or a molecular corkscrew for ubiquitinated substrates. Similar to

ubiquitin and the UPS, p97 is involved in diverse, unrelated cellular functions and pathways. Interestingly, the specificity of p97 in diverse pathways and substrates is governed by different p97 adaptor proteins (also known as p97 cofactors) that typically possess ubiquitin-binding domains (Yeung et al., 2008; Hanzelmann et al., 2011; Klopsteck et al., 2012). In general, p97 adaptors are only those proteins with p97-interacting domains or motifs, such as the UBX (ubiquitin regulatory X) domain, UBD (ubiquitin D) domain, PUB [PNGase (peptide N-glycosidase)/ubiquitin-associated] domain, SHP motif, VIP (VCP interacting protein) motif, or VBM (VCP-binding motif). UBX is the most prominent p97 interacting domain and has a similar structure to ubiquitin, but lacks sequence homology. There are at least 13 different UBX domain-containing proteins (UBX proteins) in mammalian cells, and their functions are largely uncharacterized. All UBX proteins were recently shown to interact with p97 as major regulators of p97 function (Alexandru et al., 2008). It is not known how many different p97 adaptor complexes exist in the cell, but current understanding of p97 and its adaptors suggests the existence of the following three primary mutually exclusive p97 core adaptor complexes: p97–Ufd1–Npl4 (p97⁻Ufd1–Npl4), p97–p47 (p97⁻P47) and p97–UBXD1 (p97⁻UBXD1; Meyer et al., 2012; **Figure 1B**). Each of the p97 adaptor complexes is dedicated to a specific ubiquitin-dependent cellular process. According to the “p97 core adaptor hypothesis,” the p97⁻Ufd1–Npl4 complex is involved in regulating mostly Lys48-polyubiquitinated substrates and proteasomal degradation in diverse cellular processes, such as ER-associated degradation (ERAD), chromatin-associated protein degradation (CAD), or ribosome-associated degradation (RAD). In contrast, the p97 core complexes p97⁻P47 and p97⁻UBXD1 are involved in ubiquitin-dependent proteasome-independent membrane fusion and vesicular trafficking processes. The specificity and activity of p97 core adaptor complexes to different substrates relies on a combination of secondary p97 adaptors in a process known as the p97 adaptor hierarchy (Hanzelmann et al., 2011). This is best illustrated in the p97⁻Npl4–Ufd1 complex, which processes and remodels various polyubiquitinated substrates involved in diverse pathways on the same cellular structure, such as chromatin (Alexandru et al., 2008; Verma et al., 2011; Davis et al., 2012; Mosbech et al., 2012). For example, the p97⁻Ufd1–Npl4 core complex is (i) recruited to stalled transcription by UBX4 and UBX5 (human homologs UBXD9 and UBXD7) and extracts RNA pol II from chromatin or (ii) recruited to stalled DNA replication by DNA damage-associated VCP/p97 cofactor 1 (DVC1; also known as SPARTAN or Clorf124) and extracts DNA pol delta and eta (Davis et al., 2012; Ghosal et al., 2012; Mosbech et al., 2012) or (iii) binds soluble hypoxia inducible factor 1a by UBXD7 (Alexandru et al., 2008). All of the proteins extracted, remodeled or unfolded due to p97 segregase and/or unfoldase activities are ultimately degraded by a proteasome.

In addition to its function in the UPS, in the last few years p97 function emerged in maintenance of cellular homeostasis by regulating two closely related processes, autophagy and endosomal trafficking (Ju et al., 2009; Tresse et al., 2010; Ritz et al., 2011). p97 plays an essential role in maturation of ubiquitin-coating autophagosomes, suggesting its function in autophagic degradation of ubiquitinated substrates. The focus of this review is the



function of p97 in CAD and genome stability. For details on p97 function in autophagy and endosomal trafficking, see elsewhere (Ju and Weihl, 2010b; Bug and Meyer, 2012).

CHROMATIN-ASSOCIATED PROTEIN DEGRADATION AND PROTEIN-INDUCED CHROMATIN STRESS

Chromatin is a large protein-integration platform in which structural, dynamic, and spatiotemporal association with proteins involved in diverse processes, such as transcription, DNA replication, DNA repair, and cell division, must be tightly regulated. Nearly every protein recruited to chromatin during these processes that are essential for genome maintenance require timely removal or disassembly. Various components of the UPS and proteasome are tightly associated with chromatin, particularly when an

increased protein turnover rate is needed, as in cases of DNA damage (Levy-Barda et al., 2011; Ramadan and Meerang, 2011; Butler et al., 2012). CAD plays an essential role in maintaining genome integrity and cellular homeostasis (Figure 2). Although chromatin is a key site of protein turnover necessary for genome stability, CAD has recently emerged as an important component of chromatin metabolism (Acs et al., 2011; Levy-Barda et al., 2011; Meerang et al., 2011; Feng and Chen, 2012; Galanty et al., 2012; Gudjonsson et al., 2012; Mallette et al., 2012; Mattioli et al., 2012; Ramadan, 2012; Yin et al., 2012). Many proteins that operate on chromatin are tightly bound to the chromatin structure or chromatin-associated processes and are even considered insoluble. In addition to the physical presence of proteasomes in the vicinity of chromatin, mechanical force is needed to remodel and disassemble

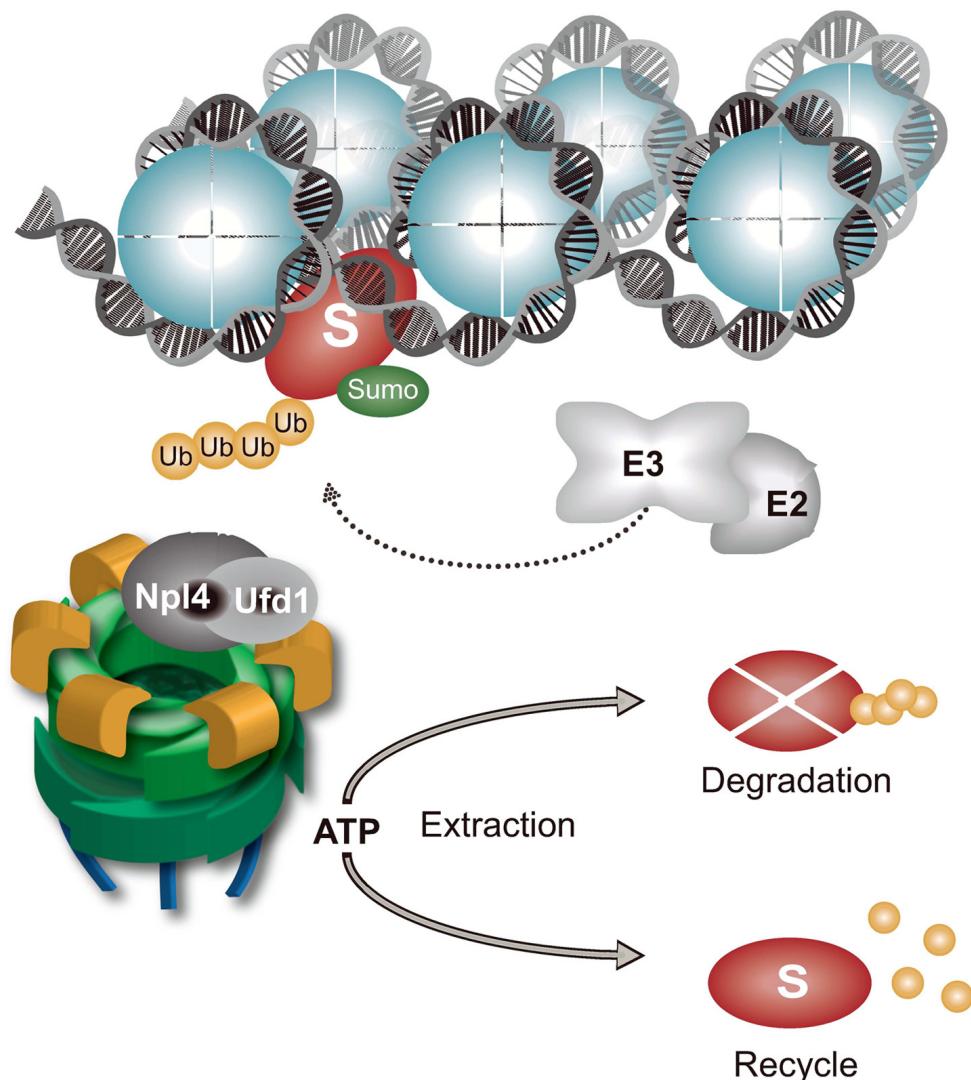
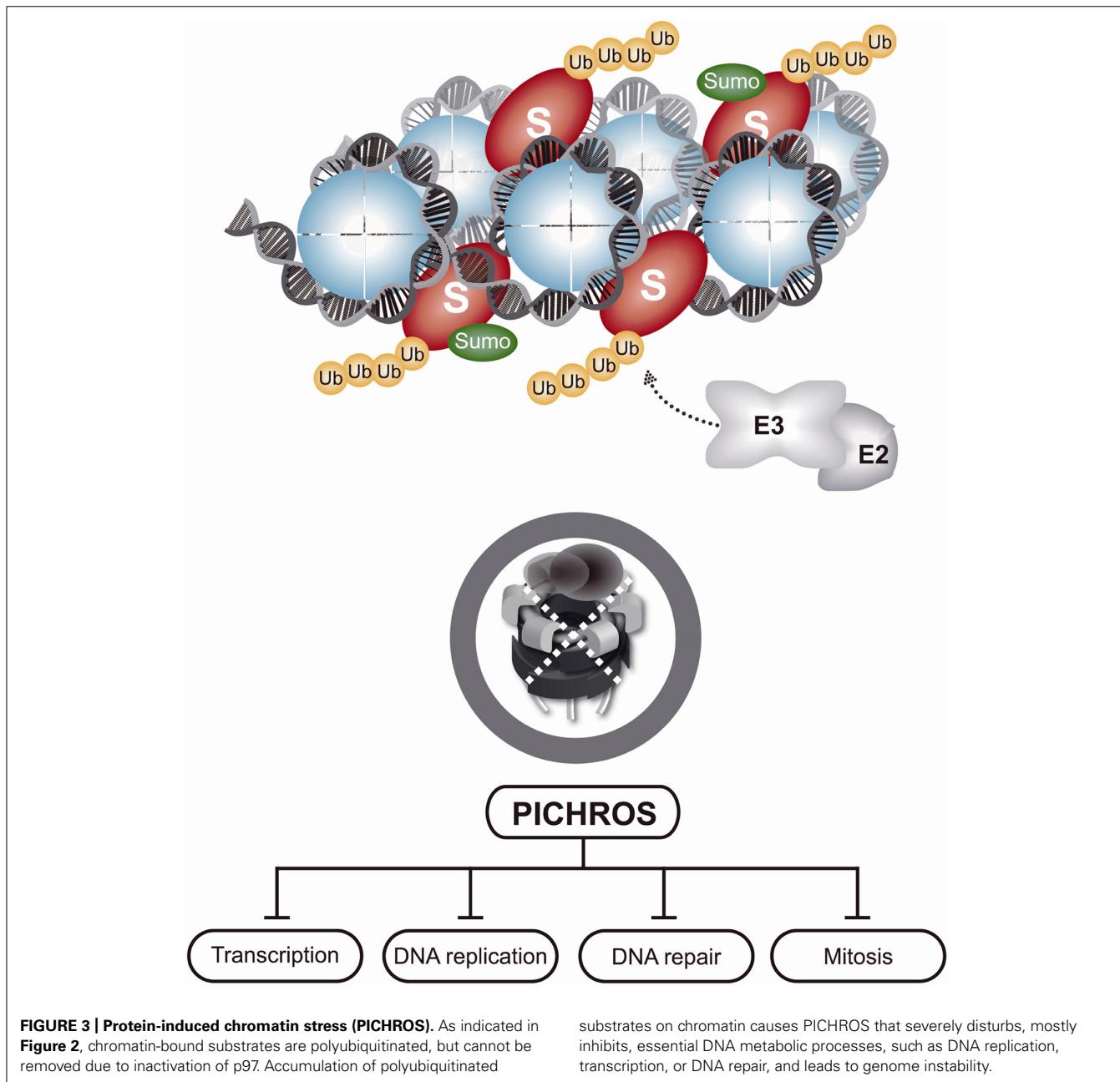


FIGURE 2 | Chromatin-associated protein degradation (CAD) regulated by p97⁻Ufd1-Npl4. Chromatin-bound proteins (S represents any known p97 substrate on chromatin) are polyubiquitinated and sumoylated by specific E3 ubiquitin and SUMO ligases. p97⁻Ufd1-Npl4 complex is recruited to polyubiquitinated substrates by a ubiquitin-binding domain in Npl4 or Ufd1.

ATP hydrolysis, located in the D2 ATPase cassette, induces conformational changes in the p97 molecule to remodel and release chromatin-bound substrates. Extracted substrates are either degraded by proteasomes or recycled. The p97⁻Ufd1-Npl4 complex maintains CAD and prevents PICHROS.

chromatin-bound substrates for final removal and degradation. Discovery of the ubiquitin-dependent molecular segregase p97 as an integral part of CAD sheds new light on how cells disassemble proteins from chromatin (Ramadan, 2012). Inactivation of p97 by RNAi or p97 segregase mutants in various systems (e.g., yeast and humans) causes K48-polyubiquitinated p97 substrates to accumulate on chromatin, leading to PICHROS. PICHROS negatively affects downstream events that involve accumulated substrates, such as DNA replication, DNA repair, mitosis, and transcription (Figure 3; Ramadan et al., 2007; Franz et al., 2011; Meerang et al., 2011; Raman et al., 2011; Verma et al., 2011). To our knowledge, the first identified chromatin-associated substrate of p97 was Aurora B (Ramadan et al., 2007). Since this discovery, information regarding removal of chromatin substrates by

ubiquitin-dependent p97 activity has rapidly progressed (Wilcox and Laney, 2009; Acs et al., 2011; Franz et al., 2011; Meerang et al., 2011; Raman et al., 2011; Davis et al., 2012; Ghosal et al., 2012; Mosbech et al., 2012). The importance of CAD function in genome stability was further confirmed by discovery of K48 ubiquitinated substrates orchestrated by E3 ubiquitin ligase RNF8 and p97 at sites of DNA damage (Meerang et al., 2011). Understanding the function of CAD in genome stability has rapidly progressed (see above). Tight collaboration between ubiquitination and sumoylation at sites of DNA damage plays a role in CAD. The evolutionary conserved SUMO-targeted E3 ubiquitin ligase (STUBL) RNF4 in mammals, Slx5-Slx8 in budding yeast and Rfp1/2-Slx8 in fission yeast is recruited to sumoylated substrates at sites of DNA damage (Prudden et al., 2007; Galanty et al., 2012; Yin et al., 2012).



STUbL polyubiquitinates SUMO-modified proteins and primes them for proteasomal degradation. Inactivation of STUbL causes hyperaccumulation of SUMO conjugates and SUMO-induced cell toxicity. In human cell lines, RNF4 is recruited to sumoylated substrates and polyubiquitinates DSB-induced factors, such as mediator of DNA damage checkpoint 1 (MDC1) and replication factor A (RPA). Consequently, polyubiquitinated MDC1 and RPA are removed from sites of damage in a proteasome-dependent manner (Shi et al., 2008; Galanty et al., 2012). RNF4-dependent MDC1 and RPA turnover allow recruitment of downstream factors, such as BRCA2 and Rad51, essential for efficient DNA repair. Inactivation of RNF4 also causes persistence of other factors at sites of damage, such as E3 ubiquitin ligases RNF8 and RNF168

and DNA damage signaling factor 53BP1 (Yin et al., 2012). Two additional E3 ubiquitin ligases, UBR5 and TRIP12, are involved in CAD and protection from PICHROS (Gudjonsson et al., 2012). UBR5 and TRIP12 regulate turnover of RNF168, the primary E3 ubiquitin ligase that ubiquitinates histones after DSBs. Depletion of UBR5 and TRIP12 causes hyperaccumulation of RNF168 and a significant increase in ubiquitin conjugates, which stimulates widespread accumulation of ubiquitin-dependent factors 53BP1 and BRCA1. Derepression of spatiotemporal SUMO- and ubiquitin-dependent protein turnover may cause hyperaccumulation of various proteins on chromatin, consequently leading to PICHROS and genome instability. In the following text, we will focus on p97-dependent CAD and PICHROS directly

related to genome stability. We will also elaborate the function of p97 in cell cycle, which may indirectly affect genome stability.

p97 IN THE CELL CYCLE

Cell cycle progression can be simplified into two main processes, DNA replication (S phase) and segregation of replicated chromosomes into two daughter cells (M phase). The G1 and G2 phases are intercalated to ensure that all requirements necessary for safe DNA replication and segregation are achieved. The transition from one cell cycle phase to another is controlled by numerous mechanisms to ensure correct cell division. Cyclin-dependent kinases (CDKs) play a central role in cell cycle progression. The activity of CDKs is coordinated by transcription and ubiquitin-dependent degradation of different cell cycle-specific cyclins, CDK inhibitors, and phosphatases (Malumbres and Barbacid, 2009). Additionally, checkpoint mechanisms activated upon DNA damage ensure the quality of DNA during replication and segregation (Branzei and Foiani, 2009).

The *Cdc48* gene (p97 homolog) was identified in the first genetic screen for cell division cycle (*cdc*) mutants in yeast (Moir et al., 1982). *Cdc48* generally attaches to the ER, but relocates in the nucleus after phosphorylation in a cell cycle-dependent manner (Madeo et al., 1998). Several observations in yeast and other organisms have revealed that *Cdc48* is crucial for normal cell cycle progression and associated genomic stability (Mouysset et al., 2008; Deichsel et al., 2009). In yeast, mutations in the *Cdc48* gene cause delayed G1/S transition and G2/M arrest. In *C. elegans* embryos, depletion of Cdc-48 or one of its cofactors, Ufd-1 or Npl-4, causes delays in S phase progression due to activation of replication checkpoints (Mouysset et al., 2008). p97 or Ufd1–Npl4 inactivation also leads to delayed progression through anaphase and exit from mitosis in human cell lines and *Xenopus* egg extracts (Ramadan et al., 2007; Dobrynin et al., 2011). These p97-defective phenotypes clearly demonstrate the crucial function of p97 in different phases of the cell cycle. p97 is important for protein degradation, and cell cycle progression requires removal/degradation of cell cycle related proteins. Determining the role of p97 in regulating these processes is important for understanding how protein recycle/degradation affects the normal cell cycle.

p97 IN G1/S TRANSITION

Many cell fate decisions are determined in the G1 phase of the cell cycle. A key question is whether or not to proliferate (replicate). When the cellular environment is favorable, cells initiate the division cycle. The cellular commitment to replicate the genome and divide is known as the restriction point. After passing the restriction point, cells switch from mitogen-dependent growth in early G1 to growth factor-independent progression in S phase, which is controlled by the retinoblastoma protein (pRb) and the cdk4/cyclin D complex. Cdk4/cyclin D hyperphosphorylates pRb, which consequently releases E2F transcription factors that activate transcription of several regulatory genes necessary for G1/S transition and S phase progression, such as cyclin E and cyclin A. The activity of cdk4/cyclin D needs to be tightly regulated (Malumbres and Barbacid, 2009).

Mutations in the *Cdc48* gene delay G1/S transition in budding yeast (Fu et al., 2003). Cdc28/Cln, a yeast Cdk1/cyclin important for G1/S transition controls the execution of Start (a yeast cell cycle commitment point equivalent to the restriction point in mammalian cells). Far1p is a Cdc28/Cln inhibitor and its degradation is needed for G1/S progression. Cdc48 physically interacts with ubiquitinated Far1p and stimulates its degradation. The defect in G1/S transition after *Cdc48* inactivation was shown to be due to persistence of the Cdc28/Cln inhibitor Far1p. In contrast to normal cells in which Far1p is degraded following release from G1 arrest, *Cdc48* mutant cells accumulated ubiquitinated Far1p. G1/S delay could be rescued following mutations in both *Cdc48* and *Far1p* genes, clearly suggesting that *Cdc48* is required for Far1p degradation. Although no data are available, a similar p97-dependent process could exist in higher eukaryotes in which CDK activity is also regulated by CDK inhibitors. In addition to Far1p degradation, *Cdc48*–Ufd1–Npl4 complex controls G1/S transition via cell wall integrity pathway mechanisms in yeast (Hsieh and Chen, 2011). The mechanisms by which *Cdc48* controls cell wall integrity have not been determined, although *Cdc48* appears to regulate Mpkl activity, which is a MAP kinase family member important for cell wall integrity, in response to stress conditions, including heat shock.

p97 IN DNA REPLICATION AND S PHASE

To divide and preserve an intact genome, cells must tightly regulate DNA replication. DNA synthesis occurs in the S phase, but preparation starts in late mitosis and G1 by loading a pre-replicative complex (pre-RC) at each origin of replication. Some pre-RCs are active, while others remain dormant. Pre-RCs consist of an origin recognition complex (ORC), cell division control protein (Cdc6), chromatin licensing and replication factor (Cdt1), and the minichromosome maintenance (MCM) helicase complex. Pre-RC is activated by phosphorylation to recruit essential replication factors, such as MCM-10, CDC-45, and the Go-Ichi-Ni-San complex (GINS), to form a pre-initiation complex (pre-IC), which recruits DNA primase and polymerases to initiate DNA synthesis. After the origins fire, the pre-RC factors are removed or inhibited to prevent re-replication of the genome during the same cell cycle. Various obstacles during DNA synthesis, such as secondary DNA structures, DNA–protein complexes or damaged bases, can stall the fork or lead to fork collapse. To ensure that the DNA replication fork can handle all of these challenges, cells activate an intra-S phase checkpoint (Branzei and Foiani, 2010).

p97 function includes regulation of DNA replication and progression through the S phase (Mouysset et al., 2008). Depletion of Cdc-48 (p97) or its adaptors Ufd-1 or Npl-4 in *C. elegans* significantly delays progression through the S phase due to activation of atl1 (ATR) and the Chk1-dependent intra-S phase checkpoint. Depletion of Cdc-48, Ufd-1, or Npl-4 reduces the number of nuclei and total amount of DNA and increases the number of chromosomal bridges in *C. elegans* embryos. Depletion of intra-S phase checkpoint kinases completely restores the delay in S phase progression in Cdc-48-, Npl-4-, or Ufd-1-defective cells, but could not restore the number of nuclei, DNA content or decrease the number of chromosomal bridges. Recent work by

two independent groups provided mechanistic insight into the role of p97^{-Ufd1-Npl4} in DNA replication (Franz et al., 2011; Raman et al., 2011). The research groups showed that p97 regulates Cdt1 chromatin turnover and stability via two distinct pathways, (i) a UV lesion-related pathway (Raman et al., 2011) and (ii) firing and elongation of the replication fork (Franz et al., 2011). In the UV lesion-related pathway, p97 regulates destruction of Cdt1 at sites of UV-induced DNA damage. After UV damage, nucleotide excision repair (NER) machinery recognizes distorted DNA (thymine dimers) and excises damaged DNA strands 25–30 nucleotides in length. This gap is repaired by DNA polymerization in proliferating cell nuclear antigen (PCNA)- and E3 ubiquitin ligase Cul4–DDB2-dependent manners. Cdt1 associates with PCNA to initiate DNA replication at sites of damage, but must be tightly regulated to prevent re-replication. After initiation of DNA synthesis, PCNA-bound Cdt1 is polyubiquitinated by Cul4–DDB2, extracted from chromatin in a p97^{-Ufd1-Npl4}-dependent manner and presented to the proteasome for final degradation (Raman et al., 2011). p97 directly controls gap-filling DNA synthesis after UV damage. In the second pathway, p97 controls DNA replication by extracting polyubiquitinated Cdt1 from chromatin under physiological conditions in *C. elegans* and a *Xenopus* egg extract. Inactivation of the p97^{-Ufd1-Npl4} complex stabilizes Cdt1 on interphase and mitotic chromatin. Consequently, Cdc45 and GINS are stabilized on chromatin-bound Cdt1 and could interfere with DNA replication fork elongation (Franz et al., 2011). Cdc-48/p97 depletion leads to accumulation of Cdt1 on chromatin in *C. elegans* embryos, *Xenopus* egg extract and human cell lines. Re-replication, a typical phenotype caused by Cdt1 over-expression, was not observed in p97/Cdc-48 depleted cells. In contrast, two independent research studies showed that inactivation of p97 caused G2/M arrest in human embryonic kidney cell line (HEK293) and decreased the total DNA content in *C. elegans* (Mouysset et al., 2008; Raman et al., 2011). This is diametrically different from a typical re-replication phenotype, which involves an increased amount of total DNA characterized by elevated Cdt1 protein levels (Teer and Dutta, 2008; Ramanathan and Ye, 2011). The molecular mechanisms involving p97 regulation of DNA replication cannot be simply attributed to extraction and stability of Cdt1 from chromatin. However, the evolutionarily conserved function of p97^{-Ufd1-Npl4} in DNA replication has been demonstrated (Mouysset et al., 2008; Deichsel et al., 2009; Franz et al., 2011; Raman et al., 2011). p97 most likely regulates multiple steps during DNA synthesis. This hypothesis could be further supported by direct physical interactions with several replicative helicases, such as the Werner protein and HIM-6 (Bloom helicase homolog; Partridge et al., 2003; Indig et al., 2004; Caruso et al., 2008).

p97 IN MITOSIS

Mitosis is characterized by profound changes in cell physiology that allow nuclear envelope disassembly and separation of genetic material into two daughter cells. Spatiotemporal coordination of complex mitotic processes depends on phosphorylation and ubiquitination events that are crucial for genome integrity. At the end of mitosis, inhibition/degradation of several kinases, such as cyclin B and Aurora B, leads to spindle disassembly, cytokinesis,

chromatin decondensation, and nuclear envelope reformation (Carmena et al., 2012).

Aurora B was the first p97 substrate discovered in chromatin (Ramadan et al., 2007). In *Xenopus* egg extracts and *C. elegans*, p97/Cdc-48^{-Ufd1-Npl4} binds polyubiquitinated Aurora B and extracts it from mitotic chromatin. p97-dependent removal of Aurora B allows chromatin decondensation and nuclear envelope formation during exit from mitosis. A similar mechanism was observed in human cell lines, although regulation of Aurora B activity by p97 initiates much earlier during mitosis (Dobrynin et al., 2011). Depletion of Ufd1 or Npl4 causes an increase in Aurora B activity, which leads to defects in chromosomal alignment in anaphase, resulting in missegregated chromosomes and multi-lobed nuclei. These results establish p97^{-Ufd1-Npl4} as a negative regulator of Aurora B activity, which regulates multistep processes in chromosome dynamics during mitosis.

Similarly, Cdc-48 in *C. elegans* was shown to be required for proper condensation and segregation of meiotic chromosomes by controlling AIR-2/Aurora B (Sasagawa et al., 2012). Cdc-48 is required for localization of AIR-2 at regions between homologous chromosomes in meiosis I. In the absence of Cdc-48, higher levels of AIR-2 increase phosphorylation of its substrates over the entire length of the chromosomes, leading to defective chromosome segregation.

In contrast to the p97^{-Ufd1-Npl4} core complex, which extracts and inactivates Aurora B in mitosis, another p97 core complex (p97^{-P47}) balances Aurora B activity in mitosis using a different mechanism (Cheng and Chen, 2010). The Cdc-48^{-Shp1} complex (p97^{-P47} in metazoans) facilitates nuclear localization of Glc7, the yeast ortholog of protein phosphatase-1 (PP1), which counteracts Aurora B kinase activity. Inactivation of the Cdc-48^{-Shp1} complex causes cell cycle arrest in metaphase due to a defect in bipolar attachment of the kinetochore that activates the spindle checkpoint.

In addition to Aurora B regulation, Cdc48/p97 is required for proper spindle disassembly at the end of mitosis (Cao et al., 2003). *Xenopus* egg extracts containing the dominant negative form of p97 (p97QQ) or depleted of Ufd1 or Npl4 cofactors were unable to disassemble the spindle and reform interphase microtubules (MTs) and remained in a mitotic state. Cdc48/p97^{-Ufd1-Npl4} specifically interacts with spindle assembly factors XMAP215, TPX2, and Plx1 at the exit of mitosis, promoting its sequestration in the cytoplasm or extraction from MTs. This function of p97 was further confirmed in yeast where p97 (Cdc48) is required for degradation of spindle assembly factors Ase1 and Cdc5.

In conclusion, two p97 core complexes, p97^{-Npl4-Ufd1} and p97^{-P47}, coordinate Aurora B activity in chromatin to allow proper chromosomal alignment, segregation, decondensation, and nuclear envelope formation at mitotic exit. In addition to direct effects on mitotic chromatin, the p97^{-Ufd1-Npl4} complex also regulates spindle disassembly at the end of mitosis.

p97 IN DNA DAMAGE

To cope with DNA damage, preserve genetic information for the next generation and survive, cells have evolved a variety of DNA repair mechanisms specific for different types of damage, which are orchestrated by DDR and DDT (Nyberg et al., 2002; Jackson

and Bartek, 2009; Curtin, 2012). Although p97 phosphorylation after IR and physical interaction with BRCA1 predicted the role of p97 in DNA repair more than a decade ago, its function in DNA repair remained a mystery (Zhang et al., 2000; Livingstone et al., 2005). The molecular mechanism of p97 emerged in DSB repair, TLS and transcription-coupled NER (TC-NER) only recently (Acs et al., 2011; Meerang et al., 2011; Verma et al., 2011; Davis et al., 2012; Ghosal et al., 2012; Mosbech et al., 2012). Whether p97 is involved in other DNA repair pathways remains to be discovered.

DVC1 LINKS p97 TO TRANSLESION DNA SYNTHESIS

DNA is particularly vulnerable to damage during DNA replication. Some base adducts may escape detection by repair proteins before progression through the S phase. During replication, these lesions cannot be accommodated at the active sites of replicative DNA pols and stall progression of the DNA replication fork (Lehmann, 2011a). Stalled replication forks activate DDT pathways and recruits translesional DNA pols, a mechanism known as TLS (Ulrich, 2007; Sale et al., 2012). TLS uses a DNA pol switch mechanism, modulated by the ubiquitination status of PCNA, in which replicative DNA pols are exchanged for translesion DNA pols (Lehmann, 2011b).

Translesion DNA synthesis is initiated by monoubiquitination of PCNA by the E3 ubiquitin ligase Rad18. Specialized translesion DNA pols are recruited to monoubiquitinated PCNA at stalled replication forks to promote bypass of damaged DNA. However, replication through a lesion site often requires the sequential action of two DNA pols in which one inserts a nucleotide opposite the damage (DNA pol eta) and the other extends from the inserted nucleotide (DNA pol zeta; Lange et al., 2011). After lesion bypass, PCNA is deubiquitinated by ubiquitin protease USP1, which stimulates the switch from translesion to replicative polymerases and continuation of DNA replication (Fox et al., 2011).

A newly identified p97 adaptor, DVC1, also known as Spartan, recently emerged as a central factor in TLS (Centore et al., 2012; Davis et al., 2012; Ghosal et al., 2012; Juhasz et al., 2012; Machida et al., 2012; Mosbech et al., 2012; Kim et al., 2013). DVC1 is conserved from *C. elegans* to humans and it localizes to UV-induced DNA lesions and other DNA replication-related damage, but not to DSBs induced by IR. DVC1 depleted cells were shown to be sensitive to various replication-related genotoxic agents. Together with physical interaction and cellular colocalization with PCNA, these findings emphasize the role of DVC1 in replication-related DNA damage processes, but not DSB repair after IR.

DVC1 contains several domains, such as a putative zinc metalloprotease domain (SprT), a p97-interacting motif (SHP), a PCNA interaction domain (PIP), and a zinc finger ubiquitin-binding domain (UBZ4), that link its function to ubiquitin-dependent and p97-regulated replication-related processes (Davis et al., 2012; Ghosal et al., 2012; Mosbech et al., 2012). In addition to interacting with PCNA, DVC1 interacts with other essential proteins involved in DNA replication, TLS, and UPS, such as DNA pol delta, eta, Rad18, ubiquitin, and the p97-Ufd1-Npl4 complex (Centore et al., 2012; Davis et al., 2012; Ghosal et al., 2012; Machida et al., 2012; Mosbech et al., 2012; Kim et al., 2013). DVC1 was shown to directly interact with p97 at the SHP domain, which is essential

for recruiting p97 to stalled replication forks. DVC1 lacking the SHP domain can colocalize with DNA lesions, but cannot recruit p97. These data suggest undisputable roles of DVC1 and p97 in TLS synthesis.

However, the exact mechanism of DVC1 function and its collaboration with p97 at stalled replication forks are still not completely understood. First, it is not clear whether recruitment of DVC1 to stalled replication forks depends on Rad18 E3 ligase. Although three research groups claim that DVC1 recruitment to UV lesions is dependent on Rad18 (Centore et al., 2012; Ghosal et al., 2012; Juhasz et al., 2012), two other research groups have reported that DVC1 recruitment does not depend on Rad18 (Davis et al., 2012; Mosbech et al., 2012). Second, two groups claim that DVC1 brings Rad18 to chromatin and enhances monoubiquitination of PCNA (Centore et al., 2012; Ghosal et al., 2012), while others did not observe this effect. Third, one research group also suggested that DVC1 is recruited to stalled replication forks by Rad18 and PCNA-monoubiquitination, protecting monoubiquitinated PCNA from deubiquitination (Juhasz et al., 2012). Fourth, two groups have reported that DVC1 recruits p97 to stalled replication forks to extract DNA pol eta and allows the switch to replicative polymerases after bypassing the UV lesion (Davis et al., 2012; Mosbech et al., 2012). In contrast, one group showed that DVC1 has an opposite function by removing replicative polymerase delta at UV lesions to allow recruitment of DNA pol eta (Ghosal et al., 2012).

In addition to above-mentioned DVC1 function in error-free TLS orchestrated by the switch between DNA pol eta and DNA pol delta, DVC1 also negatively influences error-prone TLS orchestrated by a DNA pol eta/DNA pol zeta switch supported by Rev1 and Pold3 (Kim et al., 2013). DVC1 and its SprT domain suppress Pold3 from binding to DNA pol zeta to inhibit error-prone TLS.

Even though the exact mechanistic insight of DVC1 in TLS is not yet clear, DVC1 plays a crucial role in TLS, likely by stimulating error-free TLS and inhibiting error-prone TLS. Further research will be needed to elucidate the exact function of DVC1 at stalled replication forks and involvement of p97.

p97 IN DNA DOUBLE-STRAND BREAK REPAIR AND DAMAGE RESPONSE

Double-strand break is the most deleterious DNA lesion. If not repaired, it can cause chromosomal rearrangements, deletions and genome instability or cell death. A single DSB can cause cell lethality in yeast, suggesting that it is a highly cytotoxic DNA lesion that must be immediately detected and processed (Bennett et al., 1993). It is estimated that approximately 1% of endogenous single-strand DNA breaks convert to DSBs during S phase in mammalian cells, which correlates to about 50 endogenous DSBs per cell cycle (Vilenchik and Knudson, 2003). DSBs also occur as a consequence of certain medical interventions related to diagnostics or therapy (e.g., X-ray). To prevent genome instability, ubiquitination and sumoylation have emerged as an essential PTM in regulating DDR and DSB repair.

Until recently, the RNF8/RNF168-dependent Lys63-ubiquitin chain was regarded as the sole ubiquitin-signaling pathway at sites of DSB (Doil et al., 2009; Stewart et al., 2009; Al-Hakim et al., 2010; Ulrich and Walden, 2010; Mattioli et al., 2012). This paradigm changed when p97 function was identified in DDR and

DSB repair (Acs et al., 2011; Meerang et al., 2011; Ramadan, 2012). The p97^{Ufd1–Npl4} complex is recruited to sites of DSB soon after damage occurs. Recruitment of the p97^{Ufd1–Npl4} complex to sites of DSBs strongly depends on free nuclear ubiquitin and RNF8 E3-ubiquitin ligase activity, but not E3 ligase RNF168 (Meerang et al., 2011). This suggests the presence of an RNF8/RNF168-orchestrated cascade and a sole RNF8-orchestrated cascade at DNB sites, which was confirmed using ubiquitin chain-specific Lys63 or Lys48 antibodies. While depletion of RNF8 and RNF168 completely abolished formation of the Lys63- and Lys48-ubiquitin chains at sites of DNA damage, depletion of RNF168 only eliminated Lys63-ubiquitin chain formation. Recruitment of p97 to DSB sites strongly depends on RNF8/K48-ubiquitin chain formation. Depletion of p97 or expression of p97 segregase mutants caused increased levels and persistence of Lys48-ubiquitin chains at DSB sites. These data clearly demonstrate that p97 is recruited to DSB sites via Lys48-ubiquitin chains formed by RNF8 and extracts them from chromatin. p97 processing of Lys48-ubiquitinated substrates soon after DSBs is important for recruiting signaling and repair molecules, such as 53BP1, BRCA1, and Rad51 (Meerang et al., 2011). The inability of p97-inactivated cells to process Lys48-ubiquitinated substrates at sites of DSBs severely reduces the main branches of DSB repair, homologous recombination, and non-homologous end-joining. The specific p97 substrates at sites of DNA damage and mechanisms by which p97 influences the main branches of the DSB repair pathway remain unknown. Discovery of the p97 substrate polycomb protein L3MBTL1 in the vicinity of DSBs explains why inactivation of p97 segregase activity eliminated 53BP1 protein recruitment. L3MBTL1 possesses multiple tandem Tudor domains, which enable its high affinity to bind H4Lys20me2 in undamaged chromatin (Acs et al., 2011). After DSBs occur, L3MBTL1 is removed by p97 and allows recruitment of 53BP1 by its own Tudor domain to H4Lys20me2. p97 regulation in recruiting two other essential signaling and repair proteins, BRCA1 and Rad51, remains to be investigated. These data establish previously unrecognized RNF8-Lys48 ubiquitin cascades at DSB sites, which recruit p97^{Ufd1–Npl4} segregase activity to process Lys48-ubiquitin substrates (Ramadan, 2012).

Cdc48^{–Ufd1–Npl4}, a yeast homolog of p97^{Ufd1–Npl4}, is involved in processing sumoylated and ubiquitinated substrates at sites of DNA damage (Nie et al., 2012). Yeast Ufd1 contains a SUMO-interacting motif (SIM) that non-covalently binds SUMO conjugates and recruits the Cdc48^{–Ufd1–Npl4} complex to STUbL targets. Cdc48^{–Ufd1–Npl4} cooperates with STUbL in DNA repair to reduce stalled covalent DNA topoisomerase1-DNA adducts by binding and processing sumoylated and ubiquitinated substrates at sites of DNA damage. Whether the human p97^{–Ufd1–Npl4} complex also participates in dual recognition of sumoylated and ubiquitinated substrates at sites of DNA damage is not known.

p97 IN TRANSCRIPTION-COUPLED NUCLEOTIDE EXCISION REPAIR

A wide variety of DNA helix-distorting lesions, such as UV-induced photolesions (thymine dimers) and DNA adducts induced by chemicals in transcribed DNA, block progression of the RNA pol II complex. Blocking transcription at sites of DNA damage represents a serious obstacle for DNA replication and leads

to DNA replication fork collapse. To prevent DNA replication fork collapse at sites of stalled transcription and avoid apoptosis, cells activate TC-NER, a subpathway of NER. TC-NER rapidly removes lesions from the transcribed strand and allows transcription to continue (Fousteri and Mullenders, 2008; Lehmann, 2011a). Upon induction of DNA damage by UV or UV-mimetic drugs, thymine dimers form, transcription stalls, and the TC-NER subpathway is activated. Stalled transcription recruits E3 ubiquitin ligase Cul3 to ubiquitinate the RNA pol II complex and prime it for proteasomal degradation (Chen et al., 2007; Ribat et al., 2007). It is hypothesized that the stalled RNA pol II complex shields DNA lesions and prevents access by the NER machinery. As a result, the complex must be removed and degraded. The Cdc48^{Ufd1–Npl4} complex together with Ubx4 and Ubx5 (human homologous UBXD9 and UBXD7) play an important role in UV-dependent turnover of the stalled RNA pol II complex in yeast (Verma et al., 2011). The Cdc48^{Ufd1–Npl4–Ubx4–Ubx5} complex is recruited at sites of UV-induced damage and facilitates CAD of Rpb1, the largest RNA pol II subunit. The stalled RNA pol II complex also stimulates increased proteasome recruitment at sites of UV lesions that form a tight complex with p97/Cdc48. These results suggest tight cooperation between p97/Cdc48 and proteasomes at sites of UV lesions, in the earliest step of TC-NER. Beside its involvement in the upstream step of TC-NER, p97 also operates in the gap-filling DNA synthesis, which is the final step of both NER subpathways, TC-NER and global genome-NER (GG-NER; Raman et al., 2011). During gap synthesis, p97 promotes segregation and degradation of PCNA-bound Cdt1 to prevent re-replication (Raman et al., 2011; Ramanathan and Ye, 2011). Together, these results suggest that p97 operates at different levels in both NER subpathways. It would be interesting to see whether and how p97 operates in early steps of GG-NER.

p97 AND DISEASES RELATED TO GENOME STABILITY

p97 has been implicated in the pathogenesis of many human diseases, including breast, colorectal, lung, prostate and pancreatic cancers, chronic obstructive pulmonary disease and severe emphysema, cystic fibrosis, Alpha-1-trypsin deficiency, Paget's disease of bone, and several types of neurodegenerative disorders (Vij, 2008; Haines, 2010; Min et al., 2011). Inclusion body myopathy associated with Paget's disease of bone and frontotemporal dementia (IBMPFD) is the only disorder that has been directly linked to p97 dysfunction to date (Kimonis et al., 2008). p97 mutations have been linked to 2% of isolated familial amyotrophic lateral sclerosis (ALS; Johnson et al., 2010). IBMPFD is an autosomal dominant negative inherited degenerative disorder due to a single missense mutation in p97 in humans. There are several families with IBMPFD worldwide, but the mutations are primarily located in the N-terminal region, the linker region between the N-terminal and D1 areas and the D1 region (Weihl et al., 2009). IBMPFD is characterized by disabling weakness, osteolytic lesions consistent with Paget's disease of bone and frontotemporal dementia. Accumulation of cytoplasmic and nuclear ubiquitin-positive aggregates in the tissues of patients with IBMPFD suggests that p97 processing of ubiquitinated substrates is the key mechanism involved in pathogenesis. Because p97 processes substrates for lysosomal and proteasomal degradation pathways, it is not clear which pathway

is affected in IBMPFD (Janiesch et al., 2007; Ju and Weihl, 2010a; Ritz et al., 2011).

IBMPFD patients do not suffer from genomic instability phenotypes characterized by premature aging and/or cancer development. For example, the incidence of osteosarcoma complications in Paget's disease of bone is less than 1% (Hansen et al., 2006). In contrast, proteasomal degradation plays a crucial role in various aspects of genome stability (Shi et al., 2008; Ramadan and Meerang, 2011; Galanty et al., 2012; Ramadan, 2012). Defects in lysosomal degradation rather than defects in proteasomal degradation likely contribute to the pathogenesis of IBMPFD. There is evidence to support the hypothesis that defective p97 functioning in lysosomal degradation is the primary cause of IBMPFD.

Although there is no direct proof that p97 mutations play a role in diseases associated with genome stability, we provide evidence in this review to support an essential, conserved role of p97 in genome stability from yeast to humans. In addition, p97 or its adaptors regulate proteins involved in tumorigenesis, such as Aurora B (overexpressed in cancer cells and implicated in genome stability), I κ B [potential inhibitor of the pro-survival function of nuclear factor kappa B (NF κ B)] or HIF1a (promoter of tumor angiogenesis and metastases; Asai et al., 2002; Ramadan et al., 2007; Alexandru et al., 2008; Dobrynin et al., 2011). Elevated p97 expression correlates with the progression, prognosis, and metastatic potential of many cancers (Yamamoto et al., 2003, 2004a,b,d). For example, high levels of p97 protein correlate with colorectal carcinomas (Yamamoto et al., 2004c). Low p97 expression levels have been observed in adenomas, while high levels have been shown in all metastatic tumors. Patients with tumors that express high levels of p97 show a higher recurrence rate and poorer disease-free periods and overall survival compared to patients with tumors that have low p97 expression, suggesting that high levels of p97 indicate a poor prognosis. Similar to colorectal cancer, p97 is overexpressed in non-small cell lung carcinoma (NSCLC; Yamamoto et al., 2004b). All of the data described are based on a correlation between p97 protein levels and cancer development and progression. Whether elevated p97 expression increases the degradation of growth inhibitory proteins or elevated expression is a cellular response to protein-induced stress in cancer is not known (Haines, 2010). However, inhibition of p97 by a small molecule significantly reduced NSCLC tumor growth in *in vitro* and *in vivo* models (Valle et al., 2011). This suggests that increased p97 levels may be directly responsible for tumorigenesis.

There is no evidence that mutations in p97 are related to cancer. Considering its ubiquitin-dependent functions in diverse processes related to proliferation and maintenance of protein homeostasis, one could speculate that cancer cells rely on intact p97 function.

Because p97 is essential for cell survival, alterations in its adaptors would be expected to cause cancer. Haploinsufficiency of the FAF1 gene, a member of the UBX family of adaptors, was observed in 30% of cervical carcinomas and 12.5% of mantle-cell lymphomas (Hidalgo et al., 2005; Bea et al., 2009). FAF1 protein levels are downregulated in gastric carcinomas and a large percentage of mesotheliomas (Bjorling-Poulsen et al., 2003; Altomare et al., 2009).

p97 and the FAF1 adaptor likely play important roles in cancer development, although clear molecular mechanisms have not been elucidated.

Direct evidence of p97 involvement in genome stability and cancer development was recently emerged by the discovery of a novel premature aging syndrome due to a homozygotic mutation in the p97 adaptor DVC1. DVC1 recruits p97 to stalled replication forks and is essential in preventing mutagenesis. A DVC1 mutation in novel premature aging syndrome eliminated recruitment of p97 to stalled replication forks, which consequently induced DNA replication fork collapse and an increased level of chromosomal aberrations (our unpublished results, D. Lessel et al., submitted).

CONCLUSION

As one of the most abundant cellular proteins, p97 is essential for cell development, proliferation, and growth. p97 has recently emerged as a central element in the ubiquitin system involved in both proteasomal and lysosomal degradation pathways as well as ubiquitin-dependent, degradation-independent processes. A central function of p97 enzymatic activity is to convert its own ATPase activity into remodeling activity to release (extract) and process a myriad of ubiquitinated substrates from various cellular locations. p97 plays an essential role in protein homeostasis and protection from protein stress due to accumulation of short-lived, misfolded, old, and damaged proteins. The diversity of p97 related to a myriad of ubiquitinated substrates in a variety of cellular processes is governed by multiple adaptors. An adaptor hierarchy based on a second tier of p97 adaptors primarily from the UBX family controls three core p97 adaptor complexes, p97-Ufd1-Npl4, p97-p47, and p97-UBXD1. Although the role of p97 in the cell cycle and DNA repair has been known for several decades, its function in genome stability is beginning to emerge (Figure 4). Elucidation of CAD orchestrated by p97 in DNA replication, DNA repair and discovery of DVC1 has finally established the role of p97 in genome stability. This was further demonstrated by discovery of a novel premature aging syndrome in a human due to a homozygotic mutation in the p97 adaptor DVC1. Although p97 overexpression correlates with tumor progression, inactivation of p97 or its adaptors leads to genome instability. These facts support the hypothesis that tight regulation of the p97 system is essential for genome stability and protection against cancer. Understanding how p97 protects cells from PICHROS and its function in DNA replication, repair, recombination, mitosis and the cell cycle will be essential for fully understanding the role of p97 in genome stability, aging and cancer. Many questions await further investigation, such as the identification of p97-substrates, adapters, and pathways that contribute to genome stability. p97 has a broad range of adaptors many of which possess ubiquitin-binding domains. This strongly suggests that p97 most probably binds and modulates variety of ubiquitinated substrates, besides the ones already known and described in this review, which are essential for genome stability. Hence, we have to identify other CAD-related substrates of p97, as well as the composition of p97-adaptor for specific substrates related to genome stability. Although it seems simple, the second tier of p97-adaptors and E3-ubiquitin ligases for many established pathways and substrates is still unknown, as indicated

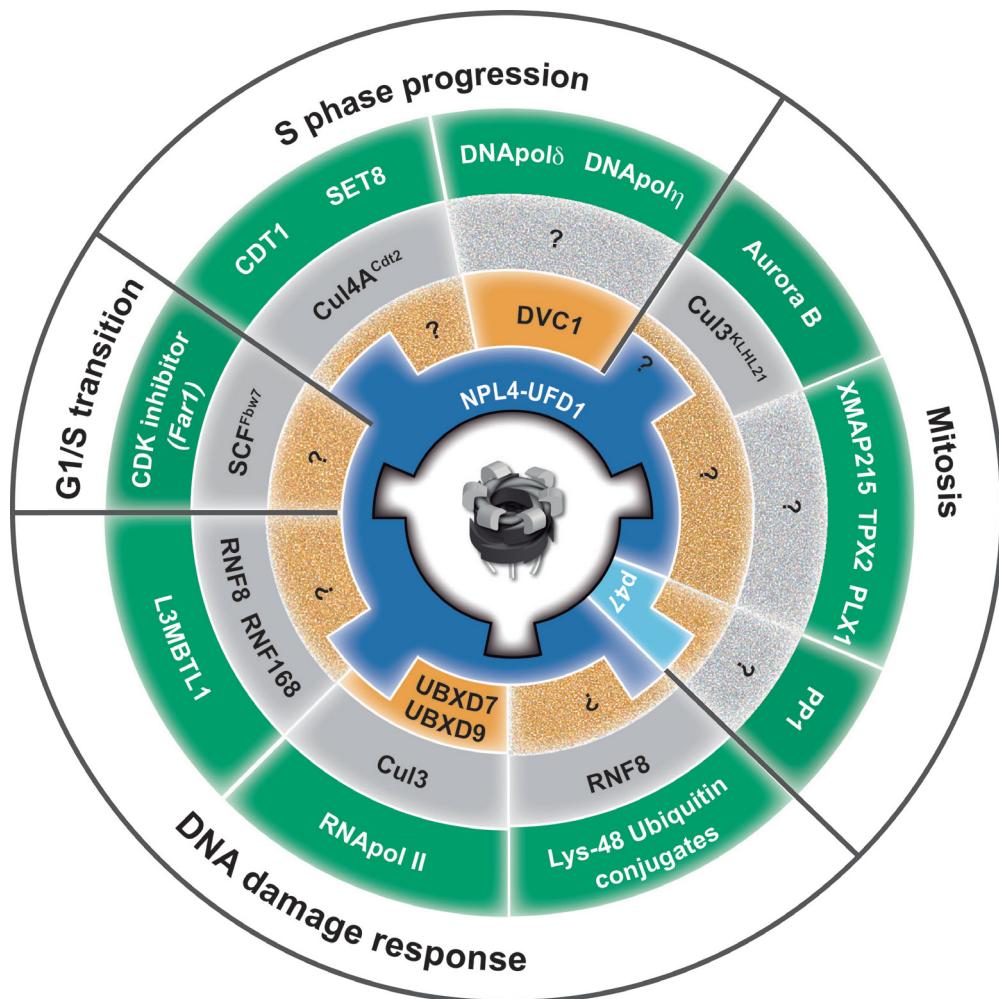


FIGURE 4 | Role of p97 in genome stability. The circle represents a summary of p97 functions in diverse cellular processes, from yeast to human, essential for maintenance of genome stability. Key players from different species are abbreviated with human homologous (e.g., yeast Cdc48 and Ubx5 is equivalent to human p97 and UBXD7). The p97 homohexamer is centrally located. The first ring (blue) represents two core adaptor complexes, Ufd1–Npl4 and p47. The second ring (orange) represents the next tier of p97 adaptors that direct the function of p97 core complexes. DVC1 directs p97–Ufd1–Npl4 function toward TLS, and UBXD7 and UBXD9 direct p97–Ufd1–Npl4 toward stalled transcription and TC-NER. The third ring (gray) represents E3 ubiquitin ligases that ubiquitinate p97-substrates. The

fourth ring (green) represents p97-substrates that must be remodeled (extracted) by p97 to avoid PICHROS and prevent genome instability. The fifth ring (white) represents different cellular processes in which p97 plays an essential role for maintenance of genome stability. PP1, protein phosphates 1/Glc7 in yeast; CDK inhibitor, cyclin-dependent kinase inhibitor Far1p in yeast; L3MBTL1, polycomb protein that contains malignant brain tumor (MBT) domain; CDT1, chromatin licensing and DNA replication factor 1; SET8, histone methyltransferase; XMAP215, processive microtubule polymerase; TPX2, microtubule associated protein; PLX1, polo-like kinase; DNA pol δ and η DNA polymerases delta and eta.

by question marks in **Figure 4**. In addition to its role in numerous cellular processes (**Figure 4**; fifth ring), p97 most probably also plays a role in other ubiquitin-controlled pathways and processes that directly regulate genome stability, such as base excision repair, mismatch repair, DNA damage checkpoints, and apoptosis. Finally, understanding the complex function of p97, its adaptors and substrates in genome stability might directly help to develop a new and more efficient anti-cancer drug(s). This speculation is based on effects of bortezomib (Velcade), a proteasome inhibitor currently used for treatment of multiple myeloma and mantle-cell lymphoma and fascinating results showing that p97 inhibitor significantly reduced NSCLC tumor growth in *in*

vitro and *in vivo* models (Valle et al., 2011). Taken together, one anticipates interesting and dynamic time ahead in investigating the function of p97 in the context of genome stability and cancer therapy.

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Pre-mRNA processing factors meet the DNA damage response

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It is well-known that DNA-damaging agents induce genome instability, but only recently have we begun to appreciate that chromosomes are fragile *per se* and frequently subject to DNA breakage. DNA replication further magnifies such fragility, because it leads to accumulation of single-stranded DNA. Recent findings suggest that chromosome fragility is similarly increased during transcription. Transcripts produced by RNA polymerase II (RNAPII) are subject to multiple processing steps, including maturation of 5' and 3' ends and splicing, followed by transport to the cytoplasm. RNA maturation starts on nascent transcripts and is mediated by a number of diverse proteins and ribonucleoprotein particles some of which are recruited cotranscriptionally through interactions with the carboxy-terminal domain of RNAPII. This coupling is thought to maximize efficiency of pre-mRNA maturation and directly impacts the choice of alternative splice sites. Mounting evidence suggests that lack of coordination among different RNA maturation steps, by perturbing the interaction of nascent transcripts with the DNA template, has deleterious effects on genome stability. Thus, in the absence of proper surveillance mechanisms, transcription could be a major source of DNA damage in cancer. Recent high-throughput screenings in human cells and budding yeast have identified several factors implicated in RNA metabolism that are targets of DNA damage checkpoint kinases: ATM (ataxia telangiectasia mutated) and ATR (ATM-Rad3 related) (Tel1 and Mec1 in budding yeast, respectively). Moreover, inactivation of various RNA processing factors induces accumulation of γH2AX foci, an early sign of DNA damage. Thus, a complex network is emerging that links DNA repair and RNA metabolism. In this review we provide a comprehensive overview of the role played by pre-mRNA processing factors in the cell response to DNA damage and in the maintenance of genome stability.

Keywords: pre-mRNA processing, RNA binding proteins, splicing, DNA damage response, checkpoint kinases

Mounting evidence collected over the last few years supports the idea that RNA-binding proteins (RBPs) involved in different steps of mRNA life, from transcription to translation, can affect genome stability programs (Matsuoka et al., 2007; Paulsen et al., 2009; Hurov et al., 2010). In particular, a number of large-scale genetic and proteomic quests for proteins involved in the DNA damage response (DDR) have revealed enrichment in RNA processing proteins, indicating that RNA metabolism and DNA repair pathways functionally intersect. However, the role played by mRNA processing factors in the cell response to endogenous and exogenous sources of DNA damage is still largely unexplored.

In this review, after a short introduction on the basic principles of pre-mRNA splicing, we will discuss genome-wide approaches implicating RBPs in the DDR. Thereafter, we focus on three particular aspects:

- 1) RNA-binding proteins may affect the splicing profiles and levels of mRNAs for proteins involved in the cell response to DNA damage. In this section, we address important facets such as (i) the role of splicing in apoptosis; (ii) the redistribution of splicing factors as a strategy to control splicing

programs after DNA damage; (iii) the modulation of mRNA stability; (iv) the importance of cotranscriptional splicing, and (v) post-translational modifications of RBPs in the DDR.

- 2) RNA-binding proteins may directly participate in the DDR. A few examples will be provided to illustrate this still poorly understood phenomenon.
- 3) RNA-binding proteins may prevent DNA damage. Once pre-mRNA has been transcribed, it is processed into mature ribonucleoprotein (mRNP) particles. In this section, we discuss the role of mRNP biogenesis factors in preventing hazardous R-loops.

Finally, we speculate on the potential role that RBPs may play in the effect of programmed DNA damage on cell differentiation, a poorly understood subject.

PRE-mRNA PROCESSING

The majority of metazoan genes consist of an ordered succession of coding (exon) and non-coding (intron) sequences. The generation of translatable mRNAs requires the precise removal of intronic sequences *via* a complex multistep reaction known as splicing. This reaction is carried out by the spliceosome, a large molecular machine, composed of five small nuclear ribonucleoproteins

(snRNPs U1, U2, U4, U5, and U6) and more than 100 different polypeptides (Wahl et al., 2009). The spliceosome recognizes short, poorly conserved, *cis*-acting sequence elements at exon–intron boundaries (5' and 3' splice sites) and uses these to remove the intron through two sequential trans-esterifications. Alternative splicing events, using various combinations of donor and acceptor sites from different exons, produce more than one mRNA molecule from a single pre-mRNA. Five distinct alternative splicing patterns have been observed: (1) cassette exons, which may be either selected or skipped during the generation of mRNA; (2) mutually exclusive exons; (3) intron retained; (4) alternative donor, and (5) acceptor sites which alter the length of exons. Moreover, alternative promoters and poly-adenylation sites contribute to the heterogeneity of transcripts encoded by a single gene (Ghigna et al., 2008). In addition to modifying protein features, alternative splicing can also affect the stability of transcripts by introducing premature STOP codons, thus directing mRNA degradation through the non-sense-mediated mRNA decay (NMD) pathway (Maquat and Carmichael, 2001). This regulatory mechanism frequently operates to control the homeostatic level of genes encoding most RBPs, particularly splicing regulators (Valacca et al., 2010). Alternatively spliced exons are usually flanked by short and degenerate splice sites, and their recognition is modulated by regulatory sequences referred to as enhancers and silencers of splicing that respectively promote and inhibit exon recognition. These elements are present both within exons (ESEs, exonic splicing enhancers and ESSs, exonic splicing silencers) and introns (ISEs, intronic splicing enhancers and ISSs, intronic splicing silencers; Black, 2003). Enhancers function by providing binding sites for serine–arginine (SR) factors, a family (about a dozen) of essential and abundant RBPs highly conserved in evolution (Cartegni et al., 2002). SR factors display multiple roles in constitutive and alternative splicing, as well as in other aspects of gene expression (Hastings and Krainer, 2001). They share a modular structure consisting of one or two copies of an RNA-recognition motif (RRM) at the N-terminus followed by a carboxy-terminal domain of variable length rich in alternating SR dipeptides (the RS domain). The RRMs determine RNA-binding specificity, whereas the RS domain mainly mediates specific protein–protein interactions that are essential for the recruitment of the splicing apparatus. In addition, RS domains are targets of phosphorylation events that influence protein interactions (Xiao and Manley, 1998), and regulate the activity and subcellular distribution of SR proteins (Gu et al., 1994; see Figure 1). Several kinases, including SR protein kinases (SRPKs) 1 and 2, Clk/Sty, dual-specificity tyrosine-regulated kinase, DNA topoisomerase I (Topo I), glycogen synthase kinase-3 and AKT/Protein Kinase B, have been shown to phosphorylate SR proteins (for a review see Ghigna et al., 2008). However, the signal transduction pathways that regulate the activity of these kinases and their role in alternative splicing are still poorly understood.

Splicing silencers may act as binding sites for factors that block splicing machinery access to a splice site. Proteins that interact with silencer elements include heterogeneous nuclear ribonucleoproteins (hnRNP), a group of RBPs that interact with RNA polymerase II (RNAPII) transcripts to form hnRNP particles (Black, 2003). Similar to SR factors, hnRNP proteins have a modular structure in which one or more RNA binding domains, generally at

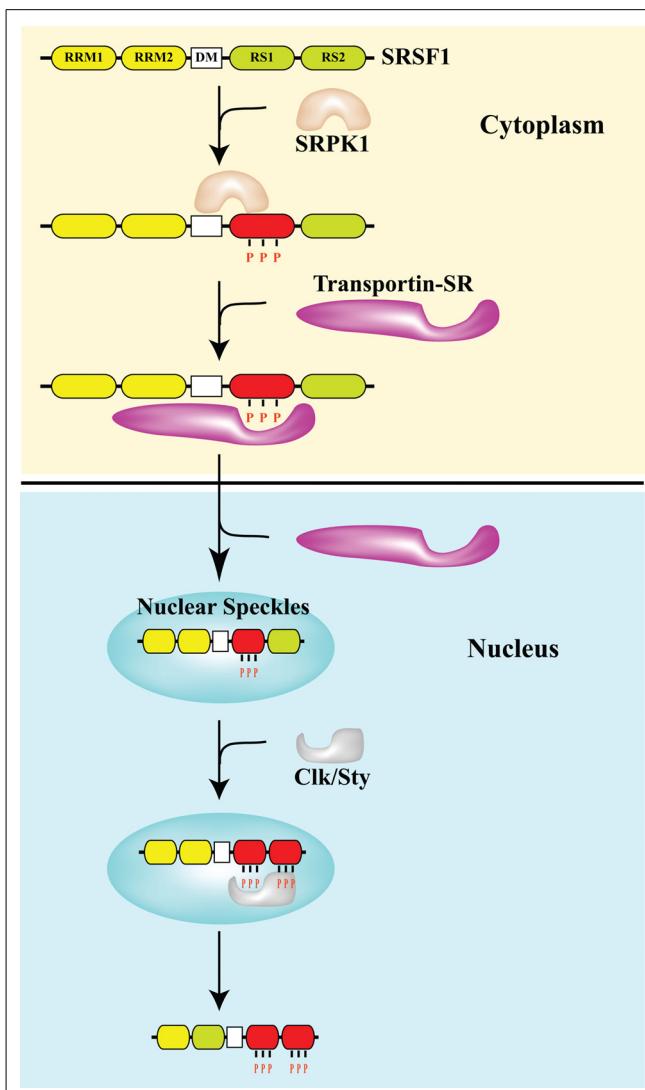


FIGURE 1 | Phosphorylation controls the subcellular distribution of splicing factor SRSF1. The arginine–serine (RS)-rich domain of the SR protein SRSF1 is phosphorylated by SR protein kinases SRPK and Clk/Sty. The docking motif (DM) restricts phosphorylation of SRSF1 by SRPK1 at the N-terminal portion of the RS domain (RS1), which is required for the interaction with transportin SR and nuclear import. In the nucleus, SRSF1 accumulates in nuclear speckles. Clk/Sty causes release of SRSF1 from speckles by phosphorylating the C-terminal portion of its RS domain (RS2; Ngo et al., 2005).

the N-terminus, are associated with different “auxiliary” domains. Three types of RNA binding domains (RRMs, hnRNP K homology – KH – domain and RGG domain, a protein region rich in Arg-Gly-Gly) have been described; these provide a certain level of RNA binding specificity (Black, 2003). The auxiliary domains are very different in sequence and control the subcellular localization and interaction with other proteins. Altogether, RNA binding specificity and protein–protein interactions contribute to the cotranscriptional assembly of hnRNP complexes that are the substrates of the splicing reaction. In addition to SR factors and hnRNP proteins, a number of tissue-specific splicing

regulators have been identified some of which, i.e., RNA binding protein fox-1 homolog (*C.elegans*) 1 and 2 (RBFOX1 and 2) and neuro-oncological ventral antigen (NOVA), bind to specific RNA sequence elements (Black, 2003).

The vast majority of alternative splicing events are controlled by the relative abundance and/or activity of widely expressed antagonistic SR factors and hnRNP proteins through a combinatorial mechanism, with multiple positive and negative factors and sequence elements influencing the final outcome of the splicing reaction. A classical example is the antagonistic activity of SRSF1 (serine/arginine-rich splicing factor 1), an SR factor, and hnRNP A1: high levels of SRSF1 induce exon inclusion, whereas high levels of hnRNP A1 promote exon skipping (Cáceres et al., 1994). Recent studies indicate that signaling pathways may control splicing decisions by affecting the subcellular distribution and/or activity of splicing regulators (Shultz et al., 2010). Many SR factors and hnRNP proteins continuously and rapidly shuttle between the nucleus and the cytoplasm (Cáceres et al., 1998), which reflects their involvement in several aspects of RNA life from transcription to translation.

Alternative splicing is a highly pervasive mechanism of gene expression regulation that affects the vast majority (more than 90%) of human genes (Pan et al., 2008). It is not surprising, therefore, that also transcripts encoding factors involved in the DDR, checkpoint or apoptosis undergo alternative splicing events that affect protein function in response to conditions of stress. However, very few genome-wide analyses on the effects of DNA damage on splicing profiles have been performed to date and we still have a very fragmented view of the logic underlying this regulatory mechanism. For instance, only recently the first comprehensive characterization of human transcriptome changes occurring in response to ionizing radiation (IR) in human lymphoblastoid cell lines was reported (Sprung et al., 2011).

LARGE-SCALE GENETIC AND PROTEOMIC ANALYSES

Several unbiased large-scale genetic and proteomic screenings in the last few years have revealed a connection between pre-mRNA processing and genome stability programs. For instance, a proteomic analysis designed to identify human and mouse proteins phosphorylated in response to DNA damage on ATM (ataxia telangiectasia mutated) and ATR (ATM-Rad3 related) consensus sites, revealed about 700 targets. Most of these belong to pathways not previously implicated in the response to DNA damage, and include factors with a role in RNA metabolism. The list of validated targets includes RBM10 (RNA binding motif protein 10), which associates with hnRNP complexes and is required for phosphorylation of the histone variant H2AX after IR (Matsuoka et al., 2007). A similar screening in yeast (Smolka et al., 2007) led to the identification of nuclear protein localization 3 (Npl3), a protein related to human SRSF1 and hnRNP A1, which is also involved in mRNA export to the cytoplasm. In the same assay, another splicing factor, called PRP19, was identified which has a direct role both in the DDR and in preventing DNA damage induced by transcription.

Another proteomic analysis quantified DNA damage-regulated changes in phosphoproteome, acetylome, and proteome in human osteosarcoma cells treated with etoposide, a topoisomerase II

(Topo II) inhibitor that causes double-stranded DNA breaks (DSBs; Beli et al., 2012). Also in this case a significant fraction of the hits corresponded to proteins involved in RNA metabolism. The same authors focused on the RNA processing factor THRAP3 (thyroid hormone receptor-associated protein 3), which is part of a multiprotein complex that controls Cyclin D1 mRNA stability, and the splicing-regulator phosphatase protein phosphatase Mg²⁺/Mn²⁺ dependent 1G (PPM1G), a nuclear member of the PP2C family of Ser/Thr phosphatases. Phosphorylation of THRAP3 mainly depends on the activity of ATR and is elicited by various DNA-damaging agents and by the DNA replication inhibitor hydroxyurea. Importantly, THRAP3 down-regulation makes cells more sensitive to fork stalling, but the molecular mechanism underlying this effect is still undefined. The lack of colocalization with γH2AX foci suggests that THRAP3 may play an indirect role in the DDR, for instance, by regulating alternative splicing or mRNA stability of transcripts for proteins involved in DDR, checkpoints or cell cycle progression.

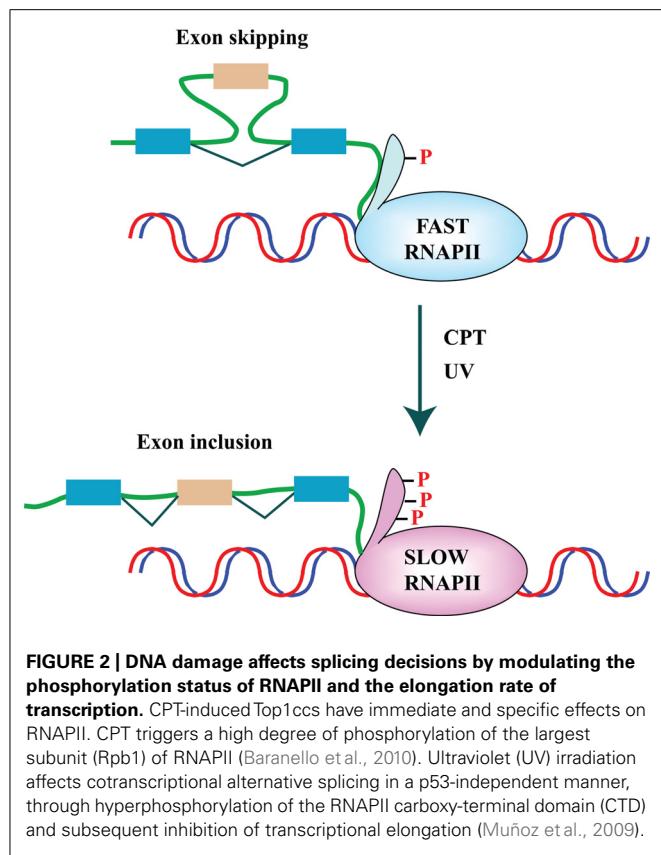
The notion that RNA processing and DNA repair functionally intersect has been recently bolstered by a genome-wide small interfering RNA (siRNA)-based screening for regulators of homologous recombination (HR; Adamson et al., 2012). This study identified a number of pre-mRNA processing proteins among positive regulators of HR, while phosphatase networks were included in the list of negative regulators.

Finally, a genome-wide approach was applied by Yves Pommier and colleagues to study the effect of the Topo I inhibitor camptothecin (CPT) on splicing decisions in human colon carcinoma HCT116 and breast carcinoma MCF7 cells. CPT preferentially affects splicing of transcripts for splicing factors, such as RBM8A, which belongs to the protein complex that tags exon-exon junctions after the splicing reaction (Solier et al., 2010). Interestingly, they showed that the production of the Topo I–DNA cleavage complex – Top1cc – triggered by CPT slows down RNA elongation through the rapid hyperphosphorylation of RNAPII and affects splicing profiles. The effect on the elongation rate of RNAPII and splicing programs appears to be an outcome shared with other DNA-damaging agents, such as ultraviolet (UV) irradiation (Muñoz et al., 2009). Two alternative models have been proposed to explain the link between the RNAPII elongation rate and splicing programs. In the “kinetic coupling model” a slow RNAPII may favor the usage of weak splice sites (de la Mata et al., 2010; Figure 2). Alternatively, hyperphosphorylation of the CTD of RNAPII may affect the recruitment of splicing factors to the transcriptional machinery as proposed in the “recruitment coupling model” (Listerman et al., 2006).

RBPs MAY AFFECT THE SPlicing PROFILES AND LEVELS OF mRNAs FOR FACTORS INVOLVED IN THE CELL RESPONSE TO DNA DAMAGE

SPlicing AND APOPTOSIS

A large amount of evidence implicates splicing decisions in the choice between cell survival and apoptosis in response to DNA damage. The functional consequence of alternative splicing on apoptosis has been documented for many genes, including cell surface receptors, such as Fas; adaptor proteins and regulators, including TRAF2 (TNF receptor-associated factor 2) and APAF-1



(apoptotic protease activating factor 1); mediators, such as B-cell lymphoma-extra (Bcl-x), Bcl-2 homologous antagonistic/killer (Bak), and myeloid cell leukemia sequence 1 (Mcl-1); and caspases. Remarkably, mRNAs encoding some members of the Bcl2 family of apoptotic factors, Bcl-x and Mcl-1, are alternatively spliced to yield both large (L) anti-apoptotic and short (S) pro-apoptotic forms. The choice between these alternatives has been investigated in several studies that reported the identification of relevant sequence elements and RBPs (reviewed in Moore et al., 2010).

Among the known inducers of apoptosis, Ceramide was the first one shown to control splicing of transcripts encoding members of the Bcl-2 (B-cell CLL/lymphoma 2) family and caspase 9. Ceramide treatment increases the level of pro-apoptotic splice variants Bcl-x(S) and caspase 9a, with a concomitant loss in the anti-apoptotic Bcl-x(L) and caspase 9b isoforms. This effect involves the regulation of the phosphorylation status of SR splicing factors, including SRSF1 (Massiello and Chalfant, 2006), through activation of PP1 phosphatase (Chalfant et al., 2002). The importance of SRSF1 phosphorylation in splicing of caspase 9 transcripts is indicated also by the observation that post-translational modification of this factor by the signaling kinase AKT promotes caspase 9b production (Shultz et al., 2010).

Insights into the molecular mechanisms that control these splicing events came from the analysis of the response to the genotoxic stress induced by oxaliplatin. This compound elicits an ATM-, CHK2 (checkpoint kinase 2)-, and p53-dependent splicing switch that favors the production of the pro-apoptotic Bcl-x(S)

variant and acts through a regulatory sequence element called SB1. Surprisingly, the same SB1 element mediates the accumulation of the larger anti-apoptotic Bcl-x(L) isoform upon activation of the PKC pathway. Thus, one splicing regulatory module can receive antagonistic signals from the PKC and the p53-dependent DNA damage response pathways to control the balance of pro- and anti-apoptotic Bcl-x splice variants (Shkreta et al., 2011) underlining the complexity of the regulatory circuits that orchestrate the cell response to conditions of stress.

In a landmark paper, Pamela Silver applied a genome scale siRNA screening to search for new regulators of Bcl-2 pre-mRNA splicing. The list of regulators identified by this screening appears to be enriched not only for splicing but also for cell cycle functions. Interestingly, treatments that induce mitotic arrest by targeting the mitotic aurora kinase A (AURKA) kinase promote the coordinated pro-apoptotic splicing of Bcl-x, Mcl1, and caspase-9 suggesting the existence of an alternative splicing network that links cell cycle control to apoptosis. Upon AURKA knockdown or inhibition, only splicing factor SRSF1 was down-regulated, most likely via modulation of post-translational turnover (Moore et al., 2010). Notably, the SRSF1 function as an inhibitor of apoptotic pathways and promoter of cell survival is in line with the oncogenic potential of this splicing regulator (Karni et al., 2007). Collectively these analyses indicate that perturbations of the post-translational modification profile and expression level of SRSF1 may impact Bcl-2 pre-mRNA resulting in the production of pro-apoptotic isoforms (Moore et al., 2010).

REDISTRIBUTION OF SPlicing FACTORS

Although SR splicing factors and hnRNP proteins are commonly considered nuclear proteins most of them continuously shuttle between the nucleus and the cytoplasm, a property that reflects their role both in mRNA export and in translation (Weighardt et al., 1995; Cáceres et al., 1998). Nuclear re-import requires the interaction with dedicated import proteins and in the case of SR factors depends on the phosphorylation of specific residues in the RS domain by SRPK1 and 2 kinases (Figure 1). In addition to being distributed throughout the nucleoplasm, SR factors display a characteristic accumulation in highly dynamic nuclear sub-compartments known as splicing speckles that are viewed as depots for proteins involved in splicing. Phosphorylation by Clk/Sty mobilizes SR factors from nuclear speckles to the nucleoplasm where transcription and mRNA maturation occurs in the perichromatin compartment (Biamonti and Cáceres, 2009). One of the strategies exploited by the cells to modulate splicing decisions in response to stress conditions, including DNA damage, is the redistribution of splicing factors. We significantly contributed to the identification of this strategy by showing that heat shock, heavy metals and osmotic stress, which threaten genome integrity, influence the sub-nuclear distribution of specific splicing factors. We have shown that splicing factors SRSF1, SRSF9, hnRNP K, Saf-B (scaffold attachment factor B), and Sam68 (Src-associated substrate in mitosis of 68 kDa) are recruited to transcription sites of repetitive genomic DNA in areas called nuclear stress bodies (Biamonti and Vourc'h, 2010). Osmotic stress also produces the accumulation of a subset of hnRNP proteins, including hnRNP A1, into cytoplasmic stress granules. This involves the

phosphorylation of hnRNP A1 by the Mnk1/2 protein kinases that act in the p38 stress-signaling pathway (Guil et al., 2006; Biamonti and Caceres, 2009). Another example of the stress effect on cellular distribution of splicing factors is hSlu7, which plays an important role in 3' splice site selection during the second step of splicing *in vitro*. It has been shown that UV irradiation decreases the nuclear concentration of hSlu7 through the modulation of its nucleus-to-cytoplasm transport. This shift is mostly dependent on the Jun N-terminal kinase cascade. Moreover, the nuclear concentration of hSlu7 affects exon choice and alternative splicing programs (Shomron et al., 2005). More recently it was shown that mitoxantrone, a Topo II inhibitor, induces relocation of several RBPs, such as TIA-1, hnRNP A1, SRSF1, and SRSF2, from the nucleoplasm to nuclear granules that serve as transcriptional factories, even though the identity of the transcribed genes has not yet been defined. This redistribution is independent of signal transduction pathways activated by DNA damage and is accompanied by changes in the alternative splicing programs of target genes such as antigen (CD44; Busà et al., 2010). Numerous other RBPs have been reported to change their distribution in response to a variety of stress conditions; however, the mechanisms involved in these redistributions have not been investigated. Thus, we still have a very superficial and fragmented description of this regulatory strategy that could be part of the cell response to DNA damage.

MODULATION OF mRNA STABILITY PROGRAMS

DNA damage elicits the activation of signaling networks, identified by apical kinases ATM and ATR, leading to the rapid phosphorylation of a large set of cellular proteins. The ultimate function is to produce an immediate arrest of the cell cycle along with recruitment of the repair machinery to damaged DNA. The list of targets for these signaling pathways also includes transcription factors, in particular p53, whose activation drives a delayed transcriptional response aimed at promoting cell cycle arrest through the induction of Cdk (cyclin-dependent kinase) inhibitors, i.e., p21, and which presides over the choice between cell survival and apoptotic pathways. A number of studies have recently identified a third intermediate branch of the DDR that operates on post-transcriptional regulatory circuits such as alternative splicing and mRNA stability programs. In this branch, RBPs would serve both as targets of the signaling network elicited by DNA damage and as transducers of signals to downstream gene expression programs.

A significant fraction of mRNAs is either up- or down-regulated after cell exposure to IR, UV, or treatment with MMS (methylmethane sulphonate; Rieger and Chu, 2004). These changes involve manipulation of mRNA stability through modulation of the interactions between RBPs and their target mRNA molecules.

One example is the mRNA encoding the growth arrest- and DNA damage-inducible GADD45 α protein, which is potently up-regulated in response to stress stimuli. Two RBPs are critical negative regulators of GADD45 α mRNA and protein levels: AUF1, which targets GADD45 α mRNA for degradation, and T-cell-restricted intracellular antigen (TIA) 1-related protein (TIAR), which prevents the association of GADD45 α mRNA

with translating polysomes. The interaction of these two proteins with the 3'-untranslated region (UTR) of the GADD45 α mRNA in HeLa cells drastically decreases after exposure to UV or treatment with MMS. Crucial for this response is the signaling pathway identified by p38 and MAPKAP kinase-2 (p38/MK2) that operates in the cytoplasm downstream of ATM and ATR. p38/MK2 modulates mRNA stability through phosphorylation of RNA-binding/regulatory proteins, including hnRNPA0, TIAR, and polyA-specific ribonuclease (PARN), and leads to stabilization of mRNAs containing AU-rich elements in their 3'-UTR (Reinhardt et al., 2010).

In addition to GADD45 α mRNA, numerous transcripts are substrates of this regulatory mechanism, a significant fraction of which encodes for proteins relevant to cell cycle control. For example HuR and hnRNP C1 bind diverse AU-rich elements in the 3'-UTR of the p21 transcript and function cooperatively to stabilize p21 mRNA in response to UV, gamma radiation, and other stress causing treatments (Cho et al., 2010). In contrast, the PCBP (poly(C)-binding protein) family of RBPs, composed of five major members hnRNP K, PCBP1, PCBP2, PCBP3, and PCBP4, binds CU-rich elements in the 3'-UTR to negatively regulate p21 expression (Waggoner et al., 2009; Scoumanne et al., 2011).

COTRANSCRIPTIONAL PROCESSING AND SPLICING

Many pre-mRNA processing factors are recruited to the RNA molecule cotranscriptionally through poorly characterized protein interactions that involve the CTD of RNAPII (Das et al., 2006). As a consequence, nascent pre-mRNA molecules emerging from the transcriptional apparatus are immediately assembled into ribonucleoprotein (RNP) complexes that constitute the substrate of the splicing reaction and determine splicing decisions. The protein moiety of these complexes depends on several parameters such as the sequence specificity of binding of splicing factors (in most cases relatively poor) and protein–protein interactions established among RBPs that are fine-tuned by post-translational modifications. Moreover, the modulation of the RBP interactions with RNAPII and the elongation rate of transcription, have been shown to play a role in splicing decisions. Although the splicing reaction does not necessarily occur cotranscriptionally, the cotranscriptional recruitment of RBPs may enhance the efficiency of the process.

In a seminal paper published a few years ago, Kornblith showed that UV affects alternative splicing profiles in a p53-independent way. This effect requires the hyperphosphorylation of the CTD of RNAPII, which leads to inhibition of transcriptional elongation, a condition known to favor inclusion of alternative exons by allowing enough time for the usage of weak splice sites (see Figure 2). Consistently, gene expression analyses with a splicing sensitive array evidenced a significant overlap between gene transcripts undergoing changes in alternative splicing after UV, and those with reduced expression (Muñoz et al., 2009).

A completely different mechanism of cotranscriptional regulation of splicing profiles has been shown to operate in response to cell treatment with the Topo I inhibitor CPT (Dutertre et al., 2010). Among the 354 exons that are skipped after a short CPT treatment, Auboeuf and colleagues focused on the splicing program of transcripts for MDM2, an E3 ubiquitin ligase that targets p53 for

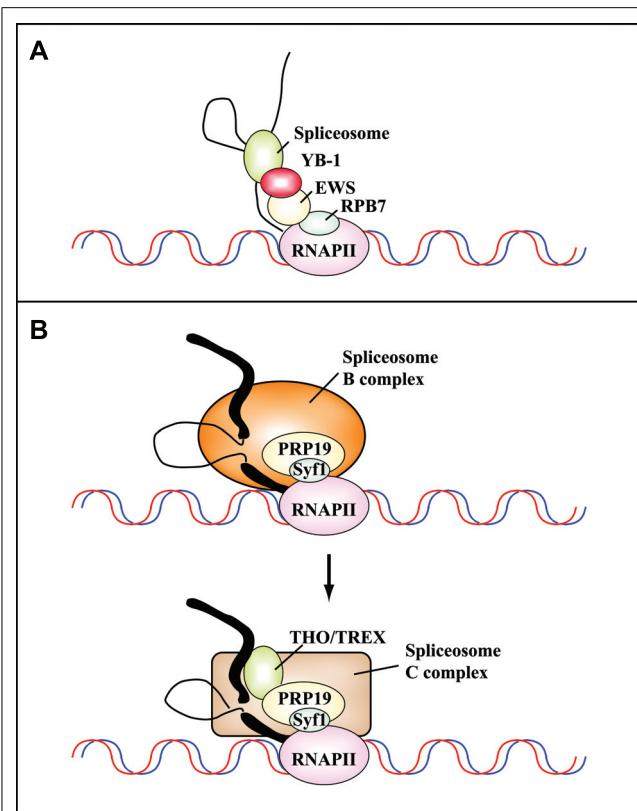


FIGURE 3 | Protein-protein interactions mediate the cotranscriptional assembly of ribonucleoprotein complexes that are targets of DNA damage-induced signaling pathways. **(A)** Schematic representation of the communication between the transcriptional and splicing machineries mediated by RPB7, EWS, and YB-1. Camptothecin inhibits the interaction between Ewing's sarcoma proto-oncogene protein (EWS), an RNAPII-associated factor, and YB-1, a spliceosome-associated factor. This results in the cotranscriptional skipping of several exons of the *MDM2* gene (Dutertre et al., 2010). **(B)** The PRP19 complex functions in transcription and is recruited to the transcription machinery by the C terminus of its component Syf1, the yeast homolog of human XAB2 (adapted from Chanarat et al., 2011). Human XAB2 co-purifies both with factors involved in transcription (RNAPII), splicing (PRP19), and TCR (XPA, CSA and CSB; Kuraoka et al., 2008). The PRP19 complex is required for the recruitment of the THO/TREX complex to nascent transcripts after the switch from the B to the C splicing complex. Thick and thin black lines represent exons and introns, respectively.

proteasomal degradation. Notably, in addition to CPT, a number of well-known genotoxic stressors, including doxorubicin and cisplatin, can promote *MDM2* exon skipping (Dutertre et al., 2010). CPT acts by disrupting the interaction between EWS (Ewing's sarcoma proto-oncogene protein), an RNAPII-associated factor, and YB-1 (Y box binding protein 1), a spliceosome-associated factor (see Figure 3A). This is the first demonstration that stress treatment can alter the communication between transcription and splicing machineries leading to exon skipping and provides a good molecular model for the rapid regulation of splicing programs in response to stress, as shown in yeast (Pleiss et al., 2007).

The EWS protein is a member of the TET family (TLS/FUS, EWS, and TAF15) of RBPs and DNA-binding proteins, and functions both in transcription and RNA processing. It is involved

in HR, the DDR, and maintenance of genome integrity and its knock-out induces a phenotype similar to that observed upon knock-out of ATM. Furthermore, the EWSR1 (Ewing sarcoma breakpoint region 1) gene product is important for resistance to IR (Hurov et al., 2010). Thus, EWS acts as a bridge between transcription and splicing machineries: it interacts with proteins involved in transcription, such as the pre-initiation complex TFIID and subunits of the RNAPII, and with splicing factors, including the U1 snRNP protein U1C, the branch-point binding protein BBP/SF1, and the spliceosome component YB-1 (see references in Dutertre et al., 2010; Paronetto et al., 2011). UV-light induces dissociation of EWS from sites of active transcription, and therefore affects the alternative splicing of genes regulated by this protein such as ABL1, CHK2, and MAP4K2 that are important for the response to cell stress and DNA damage (Paronetto et al., 2011). This is accompanied by the transient enrichment of EWS in nucleoli, which provides a further example of redistribution of factors as an efficient strategy used by the cells to reprogram gene expression after treatment with DNA-damaging agents.

POST-TRANSLATIONAL MODIFICATION OF RBPs AND DDR

Recently, we have investigated the cell response to chronic replication-dependent DNA damage in human DNA ligase I (LigI)-deficient cells. The LigI defect hampers the maturation of Okazaki fragments and results in the accumulation of single-stranded DNA breaks (SSBs) and DSBs and in the constitutive activation of the ATM checkpoint pathway (Soza et al., 2009). By applying a proteomic approach, we have shown that the entire set of SR splicing factors, particularly SRSF1, is hyper-phosphorylated in LigI-deficient cells and this modification is accompanied by a shift in the alternative splicing program of apoptotic genes such as caspase 9 (Leva et al., 2012). Notably, both the level of SRSF1 phosphorylation and splicing programs can revert to those observed in normal cells by inhibiting ATM activity, indicating that SRSF1 phosphorylation could be part of a regulatory circuit through which cells cope with DNA damage. In agreement with this interpretation, SRSF1 phosphorylation is modulated in response to a wide set of DNA-damaging insults (Leva et al., 2012) and SRSF1 is involved in the choice between pro- and anti-apoptotic pathways (Moore et al., 2010).

Phosphorylation of SRSF1 is also relevant to prevention of DNA damage induced by transcription through a process referred to as transcription-associated mutagenesis, or TAM, in which DNA damage preferentially occurs on the non-transcribed strand of DNA. Moreover, transcription can promote transcription-associated recombination (TAR), which is largely, but not exclusively (Wahba et al., 2011), due to transcription-replication conflicts generated by topological constraints. According to the twin-domain model (Liu and Wang, 1987), negative and positive supercoiling domains are transiently generated behind and ahead, respectively, of the moving transcription complex during elongation: positive supercoils can impede further DNA unwinding, whereas excessive negative supercoiling favors the opening of the duplex DNA and facilitates the hybridization of the nascent RNA molecule to the template giving rise to the so-called R-loops. One of the factors that prevents the formation of R-loops is Topo I, which relaxes super-helical stress in duplex DNA. Topo I limits

R-loop formation by targeting RNA splicing and RNP assembly factors; particularly SRSF1, which appears to function in the same pathway as Topo I in preventing replication stress (Tuduri et al., 2009). The connection between Topo I and SRSF1 was suggested for the first time in 1996 by Jamal Tazi (Rossi et al., 1996) who identified Topo I as a specific kinase for SRSF1. The kinase activity of Topo I is controlled by poly(ADP-ribose) – PAR – which shifts Topo I from SRSF1 phosphorylation to DNA cleavage. Interestingly, Topo I, SRSF1, and PAR-polymerase display a high affinity for the phosphorylated CTD of RNAPII. It has been proposed that the equilibrium between these factors is relevant both for the capacity of Topo I to relieve the torsional stress generated by RNAPII and to phosphorylate SRSF1 engaged in cotranscriptional splicing events (Malanga et al., 2008). The spacer between the two RRM s of SRSF1 appears to have an important role in this phenomenon since it controls both phosphorylation of the RS domain and DNA nicking activity of Topo I. In fact, the spacer is crucial for the positioning of RRM2 in the cavity normally occupied by DNA (Ishikawa et al., 2012). It has been proposed that this interaction may be modulated by other events that involve the spacer, namely the interaction with the mRNA export factor TAP and the methylation of two arginine residues, a post-translational modification that can also impact the subcellular localization of SRSF1 (Sinha et al., 2010).

Phosphorylation of SR factors is also relevant to modulate the splicing profile of TAF1 in response to DNA damage. TAF1 is a subunit of the general transcription factor TFIID and is required for RNAPII activity. Via alternative splicing the *Drosophila melanogaster* *TAF1* gene produces four mRNAs, TAF1-1 to 4. Interestingly, both IR and CPT promote the expression of TAF1-3 and TAF1-4 isoforms. However, the response to IR is mediated by ATM and CHK2, while the effect of CPT requires ATR and CHK1 (Katzenberger et al., 2006). The mechanism underlying this splicing decision is still unidentified. It has been proposed that AKT, a protein kinase which plays an important role in cell survival is involved. ATM mediates full activation of AKT in response to IR (Viniegra et al., 2005), and in turn AKT regulates the function of SR splicing factors by phosphorylating the RS domain (Blaustein et al., 2005).

Another example involves the regulated phosphorylation and acetylation of the SR protein SRSF2 (also called SC35). Acetylation on Lys52 in the RRM inhibits RNA binding and promotes proteasomal degradation. This modification is controlled by the competing activities of the acetyl transferase TIP60 and the deacetylase HDAC6. DNA-damaging agents such as cisplatin inhibit TIP60 expression and increase SRSF2 stability. TIP60 also controls nuclear translocation of the SR kinases SRPK1 and SRPK2, which induce phosphorylation of SR proteins and control their localization and activity. Thus, cisplatin-induced loss of TIP60 leads to the accumulation of non-acetylated, phosphorylated SRSF2, which in turn promotes the production of the pro-apoptotic splicing isoform of caspase-8 (Edmond et al., 2011). This analysis provides an exciting example of how multiple post-translational modifications and regulated proteasomal degradation of a splicing factor cooperate to promote apoptosis in response to DNA damage. Consistent with its crucial role in the activation of the apoptotic splicing program of genes such as c-flip,

caspases-8, -9, and Bcl-x, the expression of SRSF2 increases in response to DNA damage. Interestingly, SRSF1 and SRSF2 appear to have antagonistic activities with SRSF1 favoring anti-apoptotic splicing while SRSF2 promotes apoptosis. Consistent with this interpretation, SRSF2 gene transcription is controlled by E2F1, which promotes apoptosis through both transcription-dependent and -independent mechanisms (Merdzhanova et al., 2008).

In addition to phosphorylation, other post-translational modifications are relevant to activity modulation of RBPs in the DDR. An example comes from the analysis of hnRNP K, a protein crucial for IR-induced cell cycle arrest. HnRNP K cooperates with p53 in transcriptional activation of cell cycle arrest genes such as 14-3-3, GADD45, and p21, in response to DNA damage (Moumen et al., 2005). hnRNP K is a substrate of the ubiquitin E3 ligase MDM2 and, upon DNA damage, is de-ubiquitylated and sumoylated on Lys 422 in the KH3 domain. This modification is regulated by the E3 ligase polycomb Pc2/CBX4 and is required for p53 transcriptional activation. Abrogation of hnRNP K sumoylation leads to aberrant regulation of the p53 target gene p21 (Lee et al., 2012; Pelisch et al., 2012). Many other hnRNPs are SUMO substrates (Vassileva and Maturis, 2004) raising the possibility that this modification is important to modulate the activity of RBPs in response to DNA damage. For instance, sumoylation of hnRNP F could be relevant to the activity of hnRNP H/F in p53 pre-mRNA 3'-end processing, protein expression, and p53-mediated apoptosis (Decorsière et al., 2011).

RBPs MAY DIRECTLY PARTICIPATE IN THE DDR

A few RBPs have a dual life; they are associated both with complexes involved in RNA metabolism and with the DNA repair machinery. This condition reflects the fact that proteins assemblies involved in transcription, splicing, and DNA repair frequently operate on the same tract of a DNA molecule. To date no one has investigated whether the recruitment of RBPs to the DNA repair complex is evidence that RNA molecules may play a role in genome stability programs as recently suggested by the discovery of short non-coding RNAs complementary to sites of DNA damage (Francia et al., 2012). Below we report a few examples of RBPs interacting with DNA repair assemblies.

hnRNP G/RBMX

A recent genome-wide screening for regulators of HR (Adamson et al., 2012) identified hnRNP G as a positive regulator that transiently accumulates at sites of DNA damage. This finding raises the possibility (shared with other RBPs such as hnRNP C and hnRNP K) of a direct role in the DDR. The biochemical consequences of transiently accumulating hnRNP G at sites of damage remains to be determined. The authors hypothesized that the recruitment of hnRNP G could help bundle PAR (polyADP-ribose) structures and hold breaks together.

Ntr1/Spp382

Non-homologous end joining (NHEJ) in mammalian and yeast cells requires a set of common core factors, including the DNA end-binding proteins Ku70 (Ku70p) and Ku80 (Ku80p), as well as the DNA ligase LIG4 (Dnl4p) and its associated factor XRCC4 (Lif1p; Sancar et al., 2004). Recently, it has been shown that

both human XRCC4 and its yeast homologue Lif1p interact with the putatively orthologous G-patch proteins Ntr1p/Spp382p and NTR1/TFIP11 that have recently been implicated in spliceosome disassembly (Fourmann et al., 2013). G-patches are short conserved sequences of about 40 amino acids containing seven highly conserved glycine residues that have been proposed to mediate RNA binding (Aravind and Koonin, 1999). The interaction with NTR1 (Ntr1p) prevents the formation of an active enzyme complex between XRCC4 (Lif1p) and LIG4 (Dnl4p) thus reducing NHEJ efficiency (Herrmann et al., 2007).

SFPQ/PSF

SFPQ (splicing factor proline and glutamate-rich), also known as PSF (polypyrimidine tract-binding protein-associated splicing factor) and its paralogs p54nrb/non-POU domain containing octamer binding (NONO) and Paraspeckle Component 1-PSPC1 are members of the *Drosophila* behavior/human splicing (DBHS) family and components of sub-nuclear bodies called paraspeckles (Shav-Tal and Zipori, 2002). SFPQ/PSF has a direct role in the DDR that involves its ability to bind and modulate the function of RAD51 a key component of the HR pathway (Rajesh et al., 2011). Interestingly, SFPQ/PSF has DNA re-annealing and strand-invasion activity that may lead to the formation of D-loop structures similar to intermediates observed during HR (Akhmedov and Lopez, 2000). It has not yet been investigated if this protein can also interact with R-loops, which appear to be deleterious for genome stability.

SFPQ and its highly similar (71% identity) paralog NONO form a heterodimer involved in various aspects of RNA metabolism, such as transcription, pre-mRNA processing, and transcription termination. They are also implicated in nuclear retention of hyper-edited RNA (Passon et al., 2012). In this function they act together with Matrin 3 (MATR3) a highly conserved, inner nuclear matrix and RBP, which is a target of ATM and CHK1 (Blasius et al., 2011). A SFPQ/NONO complex promotes NHEJ *in vitro*, and is probably involved in DSB repair in vertebrates (Bladen et al., 2005). In agreement with this idea, attenuation of NONO expression impairs DSB repair and increases radiation-induced chromosomal aberrations (Li et al., 2009). Moreover, SFPQ/NONO is rapidly recruited to sites of DNA damage induced by laser microbeams and its release from these sites is regulated by MATR3 (Salton et al., 2010).

PRP19

PRP19/PSO4 is a multifunctional protein also known as nuclear matrix protein 200 NMP200 (Gotzmann et al., 2000), UBOX4 for its involvement in the ubiquitin pathway (Hatakeyama et al., 2001), and senescence evasion factor SNEV (Grillari et al., 2005). The PRP19 complex consists of four polypeptides that form a salt-stable core (CDC5L, PRLG1, Prp19, and SPF27) with three more loosely associated polypeptides (HSP73, CTNNBL1, and AD002; Grote et al., 2010). PRP19 is found at the core of catalytically activated spliceosomes (Grote et al., 2010) and its ubiquitin ligase activity plays a critical role in activation of the spliceosome (Song et al., 2010).

The first indication that PRP19 had a role in the DDR was the identification of the ps04-1 mutant in *S. cerevisiae* that displays

increased sensitivity to the DNA cross-linking drug psoralen. This mutant shows defects in some types of recombination, including gene conversion, crossing over, and intrachromosomal recombination. It belongs to the RAD52 epistasis group for strand-break repair and its product participates in the DNA rejoicing step of the repair of cross-link lesions (de Morais et al., 1996). In human cells, Prp19 is strongly up-regulated in response to DNA damage and its down-regulation results in DSBs, apoptosis, and reduced survival after exposure to IR. Moreover, Prp19 is a target of post-translational modifications elicited by DNA damage. The human protein is phosphorylated at S149 by ATM in response to oxidative stress and DSB-inducing agents (Dellago et al., 2012). DNA damage also induces ubiquitination of PSO4 and this modification disrupts the interaction with both CDC5L and PRLG1. Interestingly, in further support of its involvement in the DDR, the CDC5L subunit of the complex directly interacts with the master checkpoint kinase ATR (Legerski, 2009).

Recently, PRP19 has been implicated in the transcription-coupled repair (TCR) pathway, which deals with DNA damage that blocks transcription elongation. This activity of PRP19 depends on the interaction with XAB2 [xeroderma pigmentosum group A protein (XPA) binding protein 2], a molecular partner of XPA, that interacts also with Cockayne syndrome group A and B proteins (CSA and CSB) and RNAPII and it is involved both in TCR and transcription (Kuraoka et al., 2008).

Recent studies have started to uncover the intricacy of interactions between complexes once considered completely unrelated. Thus, XAB2 (also known as Syf1) mediates the interaction of PRP19 with RNAPII and is responsible for its role in TCR. In turn, the PRP19 complex is necessary for the recruitment of the THO/TREX complex to transcribed genes, which is important to prevent the formation of R-loops and genome instability. The spliceosome is a highly dynamic molecular machine that is assembled in a stepwise manner onto the pre-mRNA, leading to the formation of intermediates called complexes E, A, B, B*, and C (Wahl et al., 2009). The first trans-esterification generates the C complex, which catalyzes the second step of the splicing reaction. Interestingly, human PRP19 complexes containing XAB2/hSyf1 are present within the B complex, whereas THO/TREX components are only present in the C complex (Chanarat et al., 2011; see **Figure 3B**).

The role of RBPs in DNA repair is still largely unexplored, probably because it has been underestimated by scientists working both in the RNA and DNA repair fields. However, the list of RBPs involved in DNA repair or colocalizing with sites of DNA damage is continuously growing, which clearly points to the existence of relevant connections between the two processes. In the last decade, transcription, RNA and RNP complexes have been implicated in epigenetic processes (Burgess et al., 2012). We are tempted to propose that they may play a similar role in the profound higher-order reorganization associated with DNA repair. As a matter of fact short non-coding RNAs complementary to sequences flanking DSBs have been recently described and shown to control DDR activation at sites of DNA damage (Francia et al., 2012). Generation of these RNAs requires the activity of Drosha and Dicer two ribonucleases involved in the RNAi pathway. However, nothing is known about the nature and synthesis of the precursor RNA

molecules. Whether the RBPs listed above may have a role in this process is an open and intriguing possibility.

RBPS MAY PREVENT DNA DAMAGE

A ROLE FOR mRNP BIOGENESIS FACTORS IN PREVENTING HAZARDOUS R-LOOPS

A transcriptional R loop is a structure in which a nascent transcript is partially or completely hybridized with the template strand leaving the other strand unpaired (Huertas and Aguilera, 2003). The topology of the template DNA (i.e., accumulation of negative supercoiling behind the transcriptional apparatus), and the DNA sequence (i.e., G-richness) significantly influence the formation and size of RNA–DNA hybrids in *in vitro* reactions, suggesting that the capacity to form R-loops is an inherent property of the nascent RNA molecule. R-loops are highly mutagenic structures. The unpaired DNA strand in an R-loop, in fact, is more sensitive to DNA-damaging agents and nucleases and, as in the case of B cell immunoglobulin class switching, is targeted by activation-induced cytidine deaminase (AID)-mediated DNA cytosine deamination (Gómez-González and Aguilera, 2007). Moreover, the R-loop is highly recombinogenic and can generate a block for incoming DNA replication forks or even provide unscheduled RNA primers for DNA polymerases (Bermejo et al., 2012; **Figure 4**).

Because of their negative effects on genome stability, several mechanisms operate to avoid R-loop formation. First of all, as indicated by structural studies, the nascent transcript that emerges from the exit channel of the RNA polymerase has already separated from the template DNA strand. This also implies that R loops do not directly extend from the transcription bubble (Westover et al., 2004). Topoisomerases, mainly Topo I, are active during transcription to prevent the accumulation of negative supercoiling behind the RNAPII that are suitable for R-loop formation. Moreover, RNase H activities operate to reduce the level of RNA:DNA hybrids (Wahba et al., 2011). Finally, the nascent RNA molecule, as soon as it emerges from the transcriptional apparatus is sequestered in RNP complexes. How this protein–RNA packaging influences the capacity to form R-loops is still a matter of investigation even if it is commonly assumed that binding to RBPs is alternative to R-loop formation (Huertas and Aguilera, 2003). Notably, splicing proteins have been selected in screening performed in mammalian cells designed to identify factors able to prevent spontaneous DNA breaks (Paulsen et al., 2009). On the other hand, experimental evidence suggests that R-loops physiologically form within the cells. Indeed, R-loop structures play physiological roles in immunoglobulin class switch recombination (CSR) in human B cells (Yu et al., 2003) and in the promotion of transcription termination, as in the case of the human β -actin gene (Skourtis-Stathaki et al., 2011). Moreover they may occur during transcription of very long genes whose transcription may take more than one cell cycle (Helmrich et al., 2011).

RNA polymerase II plays a critical role in the processing of mRNA precursors (pre-mRNA). This involves the interaction of a large number of factors involved in capping, splicing and termination/poly-adenylation. The interaction is mediated by the CTD of RNAPII, which is composed mainly of a repeated heptapeptide motif, YSPTSPS, that is extensively phosphorylated

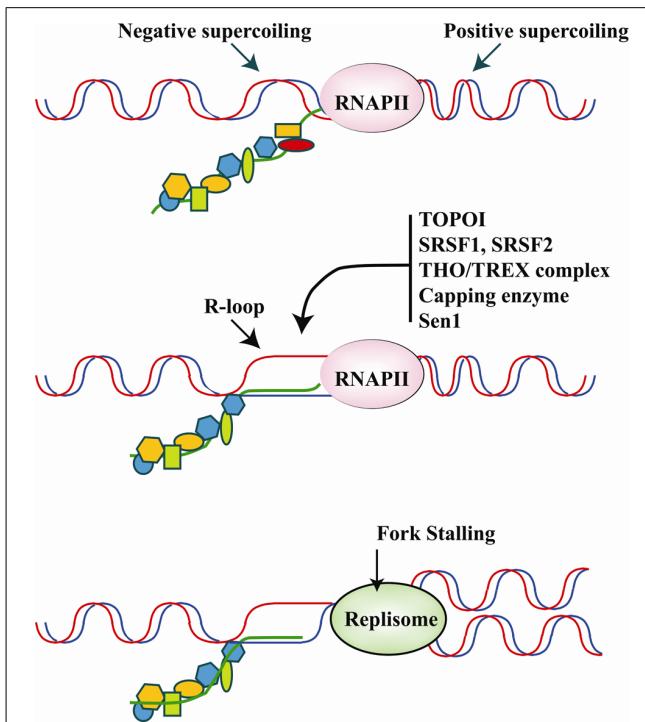


FIGURE 4 | Upper panel: the translocation of the transcriptional apparatus along the DNA induces positive and negative supercoiling, respectively, in front of and behind RNAPII. The physiological association of RBPs with the pre-mRNA molecule as it emerges from the transcriptional machinery is believed to play a major role in counteracting R-loop formation in negatively super-coiled regions. Middle panel: hybridization of the nascent RNA with the DNA template results in the formation of R-loops and occurs upon down-regulation, inhibition or mutation of several specific RBPs involved in different steps of pre-mRNA synthesis/maturation. The list of RBPs that may influence R-loop formation includes Capping enzymes, splicing factors SRSF1 and SRSF2, the THO/TREX complex, and Sen1/senataxin which is important for transcription termination. Moreover, DNA topoisomerase I (Topo I) by relieving torsional stress and phosphorylating SRSF1 can prevent R-loop formation. Bottom panel: R-loops hamper the movement of the DNA replication fork, which promotes genome instability. Thus, RBPs may be crucial to genome stability programs by inhibiting R-loop formation.

during the transcription cycle (Schroeder et al., 2000). Phosphorylation of serine-5 in the heptad repeats by human Cdk7 and yeast Kin28, occurs shortly after transcription initiation and is required for cotranscriptional recruitment of pre-mRNA processing factors. Several studies have indicated that all these factors may have a role in preventing the formation of R-loops.

Interestingly, under certain circumstances some of these factors may actually promote R-loop formation. This is the case of Capping Enzymes whose interaction with the transcription machinery is critical for RNA elongation. *In vitro*, in the presence of phosphorylated CTD, the human Capping enzymes specifically promote formation of R-loops. There is no evidence so far that Capping enzymes are involved in DNA damage. Intriguingly, however, their *in vitro* capacity to induce R-loops is antagonized by splicing factor SRSF1 (Kaneko et al., 2007), whose down-regulation *in vivo* promotes R-loop formation leading to DNA fragmentation and cell death (Li et al., 2005).

THO/TREX

One of the best-characterized RBP complexes shown to prevent R-loop formation is called THO/TREX. The multimeric THO/TREX complex is conserved throughout evolution and human homologues of all the yeast components have been identified: hTHO2/THOC2, hHpr1/THOC1, fSAP79/THOC5, fSAP35/THOC6, fSAP24/THOC7, and hTex1/THOC3, the DEAD-box RNA helicase Sub2/UAP56 and the mRNA export adaptor protein Yra1/Aly/THOC4 (Masuda et al., 2005). Both in yeast and humans the complex is functionally involved in connecting transcription, mRNP biogenesis and genome instability. Its mutation increases R-loop-dependent genome instability, and in the mouse enhances class-switching recombination in the immunoglobulin locus (Domínguez-Sánchez et al., 2011). The analysis of mutated THO/TREX in yeast by Aguilera and colleagues (Huertas and Aguilera, 2003) provided the first evidence that RNA metabolic functions have a role in preventing R-loops and that these structures mediate both impairment of transcription elongation and TAR. Using an engineered transcript containing a hammerhead ribozyme, they showed that the nascent mRNA itself has a role in the origin of transcription elongation impairment and genome instability associated with THO mutations. The function of THO/TREX-2 complexes is to couple RNAPII transcription (Gómez-González et al., 2011) with mRNA export through the nuclear envelope, a process known as gene gating. In this way another topological constraint is superimposed on to DNA during the transcription process. The ATR checkpoint phosphorylates key nucleoporins to counteract gene gating, thus neutralizing the topological tension generated when forks encounter gated genes (Bermejo et al., 2011). Mutants in some elements of this pathway may eventually lead to formation of R-loops and DNA damage. Thus, one physiological function of factors such as the THO/TREX-2 complexes would be to prevent R-loop formation and relieve topological constraints (Bermejo et al., 2012).

THSC COMPLEX

THSC (Thp1-Sac3-Sus1-Cdc31) is another complex that, similar to THO/TREX, connects transcription elongation to mRNA export via a RNA-dependent dynamic process. The THSC complex is formed by Thp1, Sac3, and Sus1 Cdc13 subunits and was previously shown to interact with the SAGA (Spt-Ada-Gcn5-Acetyltransferase) complex, a histone acetyltransferase. However, its role in transcription elongation is independent of SAGA and is linked to mRNA export. It has been proposed that a feedback mechanism exists by which improperly formed mRNPs, presumably stacked at the nuclear pore, have a backward effect promoting transcription impairment and genetic instability (González-Aguilera et al., 2008).

SPLICING FACTORS OF THE SR FAMILY

In addition to the THO/TREX complex, several mRNP biogenesis/export factors, from yeast to humans, cause TAR when mutated or down-regulated, even though their effect is weaker than that observed with THO/TREX mutants (Luna et al., 2005). Genetic studies in yeast proved that deletions of genes acting at various stages of RNA metabolism, from transcription initiation to RNA degradation and export, increase the rate of instability 4- to

16-fold over wild type (Wahba et al., 2011). In mammals, Manley's group has clearly proved that a specific subset of splicing factors of the SR family, including SRSF1 (Li et al., 2005) SRSF2 and SRSF3, can inhibit the formation of R-loops (Li and Manley, 2005). Thus, errors in RNA processing pose a major threat to genome integrity. In human and chicken DT40 cells, SRSF1 prevents R-loop formation (Li et al., 2005). A screen for suppressor(s) of SRSF1 depletion-induced genome instability in chicken DT40 cells identified RNPS1, a nuclear RBP with multiple roles in mRNA maturation. The fact that RNPS1 cannot compensate for SRSF1 function in splicing, suggests that the ability to prevent R-loops is a distinctive feature of only a few RBPs, which is separate from their activity in splicing (Li et al., 2007).

It is commonly accepted that R-loops are prevented by specific RBPs that facilitate the proper packaging of nascent mRNA into RNP particles, which in turn would strongly reduce the ability of the RNA molecule to rehybridize with the transiently opened DNA strands behind the RNAPII (Huertas and Aguilera, 2003). However, it is possible that dysfunction of mRNA processing factors may enhance R-loop formation by increasing RNA half-life, by blocking transcription elongation and possibly stabilizing negative supercoiling, or by impairing 3'-end processing and/or termination that would affect RNA release from the transcription site. Another major point of discussion concerns the mechanism through which R-loops favor genome instability. However, the majority of scientists favor the idea that R-loop-mediated genomic instability is mainly caused by impairment of replication fork progression (Tuduri et al., 2009; Gan et al., 2011). Strong support of this hypothesis comes from the analysis of Sen1/Senataxin.

Sen1/SENATAKIN

Recent data suggest that R-loops may play a physiological function in transcription termination. Two classes of terminator sequences have been identified in human genes: cotranscriptionally cleaved (CoTC) RNA sequences and transcription pause sites. The latter correspond to G-rich sequence elements and act to slow down elongating RNAPII. They have been identified in several human genes including the human β -actin gene (Skourtis-Stathaki et al., 2011). Sen1 is a conserved RNA/DNA helicase known to cooperate with Xrn2/Rat1 in promoting efficient transcriptional termination in *S. cerevisiae* (Kawauchi et al., 2008). This function is conserved in evolution. Indeed, depletion of human senataxin, the mammalian Sen1 homolog, increases RNAPII density downstream of the poly(A) site and induces R-loop formation. Taking into account the behavior of the Sen1 mutant in yeast (Mischo et al., 2011) and the effect of senataxin inactivation in human cells at the β -actin gene locus (Skourtis-Stathaki et al., 2011), it has been suggested that *in vivo* R-loops may be more common than previously believed and their unwinding by Sen1/senataxin is physiologically important for transcription termination at transcriptional pause sites. Recent data indicate that pathological R-loops in Sen1 mutants would induce hyper-recombination via inhibition of DNA replication. From this viewpoint Sen1 would be relevant to protect genome integrity from DNA damage resulting from the head-on collision of transcription and replication. This property seems to be linked to the association of Sen1 with DNA replication forks which is crucial to protect fork integrity.

across RNAPII-transcribed genes (Alzu et al., 2012). A similar role in preventing replication-transcription conflicts was proposed for human senataxin (Yüce and West, 2013). Finally, as a further example of the connections between complexes involved in pre-mRNA synthesis, RNA processing, DNA repair and replication, Sen1 has been involved also in TCR via an interaction with Rad2, the yeast homologue of human XPG (xeroderma pigmentosum complementation group G; Ursic et al., 2004).

PERSPECTIVES

DNA damage induces the activation of signaling pathways that target the expression and post-translational modification of RBPs involved in the metabolism of protein-coding transcripts. However, the physiological consequences of these events are far from being understood even though it is highly probable that targeting of RBPs may impact gene expression profiles. For instance, solid evidence bolsters the idea that the recruitment of specific splicing factors, such as SRSF1 and SRSF2, in the DDR can be linked to the inhibition/activation of apoptotic pathways. An appealing hypothesis is that this strategy could have a broader effect on gene expression and cell differentiation programs.

A growing body of data from the last ten years implicates the DDR in regulating precursor or stem cell differentiation programs (Sherman et al., 2011). One clear example is the development of vertebrate adaptive immune systems that requires the programmed induction and subsequent repair of DSBs during antigen receptor gene rearrangements to assemble a complete Ig gene *via* V(D)J recombination. The response to these programmed DSBs elicits ATM-dependent and ATM-independent mechanisms that ultimately control the expression of approximately 300 genes, a significant fraction of which regulates cellular processes important for lymphocyte development (Bredemeyer et al., 2008). Notably, several of these genes are regulated in response to genotoxic DNA damage as well, indicating that unphysiological DSBs disrupt normal cellular functions by altering specific gene expression programs (Bredemeyer et al., 2008). DSBs are also necessary for CSR and somatic hypermutation (SHM) required for the production of high-affinity antibodies of different isotypes. During CSR, production of DSBs requires the programmed formation of R-loops (Roy et al., 2008) and deoxycytidine deamination mediated by AID

(Chaudhuri et al., 2007). The response to DSBs produced by AID activates an ATM-dependent signaling pathway that regulates a network of genes involved in proliferation, B-cell self-renewal, and cell differentiation (Sherman et al., 2010). Interestingly, unscheduled AID-mediated DSBs are implicated in cancer (Park, 2012) even though it is unclear if the link with cancer involves targeting of aberrant R-loop structures.

DNA damage response may also influence differentiation of pluripotent embryonic stem cells (ESCs). DNA lesions in ESCs could be particularly harmful for the organisms. Thus, apoptotic pathways may clear severely damaged cells from the replicating stem cell pools. Alternatively, ESCs can activate a gene expression program controlled by p53 to promote cell cycle withdrawal and differentiation (Hong et al., 2009). Two parameters appear to be crucial to determine the choice between apoptotic vs. differentiation programs. The first one is related to the extent of DNA damage in the sense that apoptosis or senescence is the proper response to extensive genome-wide damage. A low level of DNA damage may induce cell differentiation programs as in developing B cells during Ig gene modifications. A second decision-regulating process is linked to the differentiation state of cells that experience DNA damage. For example, DDR signaling *via* ATM promotes the quiescence of stem cells, whereas in more advanced lineage progenitors, such as pre-B (Bredemeyer et al., 2008) cells, ATM-dependent DDR signaling promotes cell differentiation.

We speculate that, because of their involvement in gene expression programs, RBPs may have a role in all these decisions. Moreover, in view of their association with large, still poorly characterized, multiprotein assemblies that link genome stability, transcription, and pre-mRNA processing, RBPs may be crucial not only for the response to genotoxic stress but also for the programmed induction of DNA damage during cell differentiation processes.

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Posttranscriptional regulation of gene expression—adding another layer of complexity to the DNA damage response

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In response to DNA damage, cells activate a complex, kinase-based signaling network to arrest the cell cycle and allow time for DNA repair, or, if the extent of damage is beyond repair capacity, induce apoptosis. This signaling network, which is collectively referred to as the DNA damage response (DDR), is primarily thought to consist of two components—a rapid phosphorylation-driven signaling cascade that results in immediate inhibition of Cdk/cyclin complexes and a delayed transcriptional response that promotes a prolonged cell cycle arrest through the induction of Cdk inhibitors, such as p21. In recent years a third layer of complexity has emerged that involves potent posttranscriptional regulatory mechanisms that control the cellular response to DNA damage. Although much has been written on the relevance of the DDR in cancer and on the post-transcriptional role of microRNAs (miRs) in cancer, the post-transcriptional regulation of the DDR by non-coding RNAs and RNA-binding proteins (RBPs) still remains elusive in large parts. Here, we review the recent developments in this exciting new area of research in the cellular response to genotoxic stress. We put specific emphasis on the role of RBPs and the control of their function through DNA damage-activated protein kinases.

Keywords: MAPKAP-kinase 2, HuR, hnRNP A0, TIAR, PARN, DNA damage response, cell cycle checkpoint

CELLS ACTIVATE A COMPLEX SIGNALING NETWORK IN RESPONSE TO DNA DAMAGE

All life on earth must resist a constant assault on its genomic integrity by various endogenous and exogenous sources. Stalled replication forks or incomplete DNA replication during S-phase, and a plethora of different DNA lesions, such as those ubiquitously induced by UV, ionizing radiation (IR), or reactive oxygen species, as well as those intentionally provoked by treatment with chemotherapeutic agents, or radiation therapy used in cancer patients, activate a complex, kinase-based signaling network, which is collectively referred to as the DNA damage response (DDR). Activation of the DDR network through genotoxic lesions triggers signal transduction cascades to activate cell cycle checkpoints, which prevent further progression through the cell cycle as long as the lesions persist (Jackson and Bartek, 2009).

The DDR can be subdivided into two major kinase signaling branches: the ATM pathway, acting through the downstream effector kinase Chk2 and the proximal DDR kinase ATR, acting through Chk1. Some crosstalk exists between the ATM/Chk2 and ATR/Chk1 pathways, particularly when signaling through one pathway is partially or totally deficient (Kastan and Lim, 2000; Abraham, 2001; Shiloh, 2001, 2003; Bartek and Lukas, 2003). Normally however, the pathways appear to have distinct functions with only partial functional overlap in response to particular forms of DNA damage, especially at later stages in the cell cycle (Jazayeri et al., 2006). Different types of genotoxic

stress are preferentially channeled through one or the other of these two pathways. The ATM/Chk2 pathway is activated primarily in response to DNA double strand breaks (DSBs), such as those formed by IR or topoisomerase-2 inhibitors, such as etoposide or doxorubicin, while the ATR/Chk1 pathway is activated by bulky DNA lesions induced by UV and in response to replication fork collapse during S-phase (Zhou and Elledge, 2000; Abraham, 2001).

A major target of both the ATM/Chk2 and the ATR/Chk1 branch of the DDR are members of the Cdc25 family of dual specificity phosphatases. Phosphorylation-dependent inhibition of Cdc25 prevents activation of the Cdk-cyclin complexes that mediate transition from G₁ into S-phase, progression through S-phase and mitotic entry, thus establishing G₁, intra-S-phase, and G₂/M cell cycle checkpoints (Donzelli and Draetta, 2003; Rudolph, 2007). Cdc25A is required for activation of Cdk2-Cyclin E and A complexes that govern S-phase entry and progression. Chk1-mediated phosphorylation of Cdc25A creates a phosphodegron motif, resulting in SCF^{B-TrCP}-dependent ubiquitination and subsequent proteasomal degradation, as the major mechanism of inhibition (Jin et al., 2003). Cdc25B and C are required for activation of Cdk1-cyclin B complexes mediating mitotic entry. Upon DNA damage Chk1 and 2 phosphorylate Cdc25B and C, creating phosphoepitopes that are recognized and bound by phosphopeptide-binding 14-3-3 proteins (Donzelli and Draetta, 2003; Harper and Elledge, 2007). 14-3-3 serves as a molecular

chauffeur resulting in cytoplasmic translocation and sequestration of the complexes, preventing Cdc25B/C from activating Cdk1-cyclin B complexes.

We and others have recently identified a third cell cycle checkpoint effector kinase pathway that is governed by p38 α/β -dependent activation of MK2 (Bulavin et al., 2001; Manke et al., 2005; Raman et al., 2007; Reinhardt et al., 2007, 2010; Reinhardt and Yaffe, 2009). This pathway is activated in response to UV and the commonly used chemotherapeutic drugs cisplatin, camptothecin and doxorubicin (Manke et al., 2005; Raman et al., 2007; Reinhardt et al., 2007). We showed that ATM and ATR are required to activate the p38/MK2 module after doxorubicin and cisplatin (Reinhardt et al., 2007). In a series of experiments, we showed that MK2 functions as a downstream checkpoint effector kinase that is critical for cellular survival following DNA damage, specifically in cells and tumors that had lost the prominent tumor suppressor p53 (Reinhardt et al., 2007, 2009; Reinhardt and Yaffe, 2009). MK2 is required to prevent G₁/S, intra-S phase and G₂/M transition after cisplatin and doxorubicin in p53-deficient cells (Reinhardt et al., 2007). Intriguingly, MK2 appears to operate in a pathway that is redundant with, but independent of Chk1 (Manke et al., 2005; Reinhardt et al., 2007). Using oriented peptide library screening (OPLS), we determined the amino acid specificity for MK2 phosphorylation and found that it is identical to the optimal sequences selected by the checkpoint kinases Chk1 and Chk2 (Manke et al., 2005; Reinhardt et al., 2007). This finding suggested that all three kinases might share a pool of common substrates. Indeed, we could show that MK2 directly phosphorylates Cdc25A and is required for its DNA damage-dependent degradation, resulting in a G₁/S arrest after cisplatin and UV (Manke et al., 2005; Reinhardt et al., 2007). In response to doxorubicin, MK2 phosphorylates Cdc25B and C on known Chk1 sites, generating functional 14-3-3 binding sites and resulting in a G₂/M arrest (Reinhardt et al., 2007). These results suggest that cells lacking a functional p53 response recruit a general stress response network—p38/MK2—to arrest the cell cycle after genotoxic stress. More importantly, this requirement for the p38/MK2 network in p53-deficient tumors, rationalizes the use of MK2 inhibitors as chemosensitizing agents that are based on the synthetic lethal interaction between the corresponding genes *TP53* and *MAPKAPK2* (Reinhardt et al., 2009).

In addition to the activation of this canonical DDR kinase network, which brings about numerous changes in the cellular signaling circuitry occur as a consequence of posttranslational modifications of proteins functioning within the DDR network through phosphorylation, ubiquitylation or sumoylation (Reinhardt and Yaffe, 2009), the pattern of mRNA expression also undergoes significant changes after DNA damage (Rieger and Chu, 2004; Reinhardt et al., 2011). For instance, human lymphoblastoid cells from healthy adults display up- or down-regulation of thousands of mRNAs following exposure to IR or ultraviolet light (Rieger and Chu, 2004). Furthermore, transcriptome analysis following MMS or IR treatment showed that the expression levels of as much as 20% of genes in budding yeast showed a 2-fold or greater change (Gasch et al., 2001). These profound transcriptome alterations appear counterintuitive at first glance, as *de novo* transcription of genes shortly after

the infliction of DNA damage might pose a certain threat. The template DNA strand used for transcription might be damaged, leading to the transcription of potentially mutated RNA. In addition, the transcription process is energy-intensive (synthesis of an RNA molecule with n bases requires at least n NTP molecules) and relatively time-consuming. Specifically, the temporal component imposes a pivotal risk, if the protein product derived from the transcribed mRNA was rapidly needed for cell cycle arrest, DNA repair or the induction of apoptosis. Perhaps not surprisingly, DNA damage, such as that induced by UV-C irradiation, has been shown to trigger a transient repression of transcriptional activity in eukaryotic cells (Vichi et al., 1997; Rockx et al., 2000). Several molecular mechanisms have been implicated in mediating this DNA damage-induced global repression of transcriptional activity. RNA Pol II becomes hyperphosphorylated in response to genotoxic stress and is thus prevented from entering pre-initiation complexes at promoter sites (Rockx et al., 2000; Svejstrup, 2002). Furthermore, *in vitro* evidence suggests that the TATA-binding protein TBP is sequestered onto damaged DNA, reducing its availability for transcription (Vichi et al., 1997; Svejstrup, 2002). The transcriptional repression that is mediated through these molecular pathways varies depending on the type and intensity of DNA damage and is reverted upon completion of DNA repair (Svejstrup, 2002). However, this DNA damage-induced repression of transcriptional activity immediately poses the question how cells accomplish the DNA damage-induced changes in mRNA expression, which have clearly been demonstrated by numerous groups?

POSTTRANSCRIPTIONAL REGULATION OF THE DNA DAMAGE RESPONSE

As transcription is globally repressed upon DNA damage, additional mechanisms that regulate protein biosynthesis from pre-existing pools of mRNA become critically important to allow an appropriate cellular DDR. Two posttranscriptional regulatory mechanisms are at play to control protein expression following genotoxic stress: (1) selective mRNA stabilization or decay and (2) regulation of translation. Both of these mechanisms critically hinge on the function of RNA-binding proteins (RBPs) and non-coding RNAs, which modulate mRNA stability, transport and translatability through direct interactions with their client mRNAs. Thus, in addition to a well-studied plethora of post-translational modifications, including phosphorylation, ubiquitination, methylation, acetylation, and others (Harper and Elledge, 2007; Jackson and Bartek, 2009), posttranscriptional control mechanisms are emerging as a new layer of regulation within the complex DDR signaling network.

Intriguing in this regard is data that emerged from a recent phospho-proteomic screen aiming to identify novel ATM/ATR/DNA-PK substrates. The largest subset of substrates identified in these experiments were proteins linked to RNA and DNA metabolism, and specifically proteins involved in posttranscriptional mRNA regulation (Matsuoka et al., 2007). In addition, gene products responsible for nucleic acid metabolism, particularly those involved in mRNA binding and processing, have recently been identified as the largest subset of “hits” in an RNAi-mediated loss of function screen to identify modulators of DNA damage

signaling (Paulsen et al., 2009). Furthermore, data provided by Gorospe and co-workers re-enforced the role of posttranscriptional regulatory circuits in the control of a large fraction of the transcriptome in response to genotoxic stress (Fan et al., 2002). Specifically, cDNA expression arrays were employed to gauge the relative contribution of transcription and mRNA turnover to overall changes in gene expression after a variety of cellular stresses, including UV-C irradiation. In essence, a comparison of cDNA hybridization patterns of newly transcribed mRNAs derived from nuclear run-on assays, and steady state mRNA pools derived from whole cell lysates was performed. These experiments revealed that approximately 50% of the changes in mRNA steady state levels that were observed after cellular stress, were attributable to mRNA turnover (stabilization/decay), while the remaining ~50% were due to altered transcription. Lastly, applying a mass spectrometry-based interactome screen, Yaffe and colleagues identified proteins involved in mRNA splicing and translation as the largest group of molecules interacting with the critical DDR protein 14-3-3 (Wilker et al., 2007). These coinciding observations, observed in very different experimental settings, highlight the potential importance of posttranscriptional regulatory mechanisms in the context of DDR signaling, and strongly argue that the DDR may extend substantially beyond the classical ATM/Chk2 and ATR/Chk1 signaling cascades detailed above.

The first links between the kinase-based canonical DDR and posttranscriptional regulatory mechanisms were established through the study of p21^{Cip1/WAF} mRNA. p21^{Cip1/WAF} is a canonical p53 target gene and is potently induced in response to genotoxic stress (el-Deiry et al., 1993). Not only could Wang et al. show that the RBP HuR (human antigen R or ELAVL1, a member of the embryonic lethal abnormal vision-like family) formed a ribonucleoprotein (RNP) complex with p21^{Cip1/WAF} mRNA in RKO colorectal carcinoma cells following UV-C irradiation, but also that this complex formation appeared to be critical for p21^{Cip1/WAF} mRNA stabilization following genotoxic stress, as HuR depletion impaired p21^{Cip1/WAF} mRNA induction after UV-C (Wang et al., 2000). Further, the laboratory of A. Nebreda recently showed that p38MAPK induces p21^{Cip1/WAF} mRNA stabilization without significantly affecting transcription of p21^{Cip1/WAF} (Lafarga et al., 2009). p38MAPK-mediated phosphorylation of HuR on Thr-118 in response to IR was shown to be critical for cytoplasmic accumulation of HuR, enhanced binding to the p21^{Cip1/WAF} mRNA and subsequent p21^{Cip1/WAF} mRNA and protein accumulation (Lafarga et al., 2009). Further experiments revealed that the shuttling of HuR between the nucleus and the cytoplasm is tightly regulated by a variety of kinases, including Cdk1, Chk2, and MK2 (Tran et al., 2003; Abdelmohsen et al., 2007; Kim et al., 2008). recently suggested that Chk2, which shares substrate homology with MK2 (Manke et al., 2005), phosphorylates HuR on Ser-88, Ser-100, and Thr-118. This interaction is likely to occur in the nucleus, since Chk2 and HuR could be co-immunoprecipitated only from nuclear extracts (Abdelmohsen et al., 2007). Phosphorylation, particularly on Ser-100 in response to genotoxic H₂O₂, decreased the binding affinity of HuR to its target mRNA SIRT1 (Sirtuin 1), resulting in destabilization of SIRT1 mRNA, decreased SIRT1 protein levels and increased sensitivity of WI-38 human diploid fibroblasts to the cytotoxic

effects of H₂O₂. Mutation of Ser-88 and Thr-118 to Ala reduced SIRT1 mRNA binding even in the absence of H₂O₂, suggesting that phosphorylation on these sites actually promotes HuR RNP formation. It is, however, also conceivable that these particular mutations induce conformational changes that preclude effective RNA binding, since these residues are located within the RNA recognition motifs (RRMs) of HuR. Interestingly, treatment of WI-38 cells with H₂O₂ revealed that binding of wildtype HuR differed according to the target mRNA: binding of p21^{Cip1/WAF} mRNA was increased, while decreased on SIRT1 and numerous cyclin mRNAs. However, mutation of Ser-100 to Ala generally increased the binding affinity of HuR to all mRNAs tested. These observations suggest that although Chk2 is clearly activated by H₂O₂, this activity does not translate into a uniform decrease in HuR binding affinity to its target mRNAs. One could speculate that structural features within the HuR target mRNAs or the recruitment of other RBPs into the HuR RNPs ultimately dictate the affinity of HuR to its target mRNAs. It is also possible that the Chk2 recognition motif in HuR might be masked in certain RNPs, which could preclude Chk2-mediated phosphorylation of Ser-100 in certain RNPs. These questions await further clarification.

As a member of the ELAV-like family of RBPs, HuR has strong binding affinity to mRNAs that contain so-called AU-rich elements (AREs) in their 3' UTR (Dean et al., 2004). AREs act as potent mRNA destabilizing elements that target mRNA for rapid deadenylation (Chen and Shyu, 1994; Xu et al., 1997; Wilson and Treisman, 1988). AREs can be subdivided into three classes: class I and II AREs contain copies of an AUUUA pentameric repeat, called Shaw-Kamen motif (Shaw and Kamen, 1986). Class I AREs contain 1–3 scattered Shaw-Kamen motifs in the 3' UTR, class II AREs contain multiple, partially overlapping AREs in their 3' UTR, and class III AREs commonly lack the AUUUA pentamer, but are enriched for U-rich sequence stretches (Dean et al., 2004).

Nagamine and colleagues (Tran et al., 2003) showed that HuR binds and stabilizes the urokinase plasminogen activator (uPA) mRNA in an ARE-dependent manner. The authors went on to show that overexpression of constitutively active MK2 resulted in stabilization of ARE-containing reporter mRNAs. This effect correlated with an MK2-dependent cytoplasmic accumulation of HuR. Furthermore, treatment with H₂O₂, a known MK2 activating stimulus, also resulted in cytoplasmic HuR accumulation. The authors demonstrated that increased binding of HuR to ARE-containing uPA mRNA and stabilization of an ARE-containing reporter mRNA in response to H₂O₂ depended on MK2 acting downstream of p38MAPK. However, no evidence suggesting that MK2 directly phosphorylates HuR in this system was presented in this study.

In contrast to the molecular effect of p38MAPK, Chk2, and MK2, Cdk1-mediated HuR phosphorylation on Ser-202 was recently shown to sequester HuR in the nucleus (Kim et al., 2008). Cdk1 inhibition promoted a cytoplasmic accumulation of HuR, while a predominantly nuclear localization of HuR was observed under conditions of high Cdk1 activity. Furthermore, a Ser-202 to Ala mutant form of HuR was located primarily in the cytoplasm, while phospho-Ser-202 HuR could be detected almost exclusively in the nucleus. Kim et al. further showed that Cdk1-dependent

Ser-202 phosphorylation of HuR was essential for 14-3-3 δ binding to HuR. However, it was never formally demonstrated that the phosphopeptide-binding protein 14-3-3 δ directly binds a phosphoepitope surrounding Ser-202.

Among the known DDR kinases, the p38MAPK/MK2 signaling complex probably has the strongest ties to posttranscriptional control of gene expression. Anderson and colleagues characterized the MK2-mediated regulation of the zinc finger protein Tristetraprolin (TTP), which had been shown to bind and destabilize ARE-containing mRNAs such as TNF α (Stoecklin et al., 2004). ARE-containing mRNAs are unstable under normal conditions and are stabilized in response to various cellular stressors, such as UV, lipopolysaccharides (LPS), or arsenite (Kedersha and Anderson, 2002). In their experiments, Anderson and colleagues showed that MK2-mediated phosphorylation of TTP on Ser-52 and Ser-178 in response to arsenite generated a phosphoepitope that was subsequently engaged by 14-3-3 (Stoecklin et al., 2004). TTP binds to ARE-containing target mRNAs and directs them to exosome-dependent degradation. TTP:14-3-3 complex formation resulted in exclusion from stress granules (SGs) and inhibition of TTP-dependent degradation of ARE-containing β -globin reporter mRNA. SGs are the morphological correlate of an abrupt, stress-induced translational arrest resulting in rapid polyribosome disassembly (Kedersha and Anderson, 2002). These cytoplasmic granules consist of a number of proteins involved in RNA metabolism, as well as stalled initiation complexes, which are bound to numerous mRNAs (Anderson and Kedersha, 2006). The mRNA molecules from disassembled, stalled polyribosomes are sorted into SGs where the fate of each individual messenger is determined by RBPs that either promote RNA stabilization or decay (Kedersha and Anderson, 2002; Kedersha et al., 2005). SG proteins, such as TIA-1 and HuR, bind to ARE-containing mRNAs, and control their stability and translation (Anderson and Kedersha, 2002, 2006; Kedersha and Anderson, 2002; Kedersha et al., 2005). As an alternative mechanism to TTP:14-3-3 complex formation, it could be shown that phosphorylation of TTP by MK2 blocks mRNA decay by inhibiting the recruitment of the CCR4-CAF1 deadenylase complex (Marchese et al., 2010).

Like TTP, BRF1, subunit of the RNA polymerase III, is an ARE-binding protein that has recently been shown to be a direct substrate of MK2. Phosphorylation of BRF1 on four distinct residues (Ser-54, Ser-92, Ser-203, and an unidentified site in the C-terminus) reduced the ability of BRF1 to promote ARE-mediated decay. However, the mechanistic details of this effect remain somewhat unclear (Maitra et al., 2008).

Besides TTP and BRF1, which promote ARE-mediated decay, MK2 has also been shown to directly phosphorylate hnRNP A0, a protein that specifically interacts with ARE-containing mRNAs, exerting a stabilizing effect on its RNA targets. Rousseau et al. (2002) identified hnRNP A0 (heterogeneous nuclear RNP A0) as a protein with binding affinity for the AREs in the 3' UTR of TNF α in macrophage lysates. They further showed that MK2 phosphorylates hnRNP A0 on Ser-84 following LPS treatment. Pharmacological inhibition of p38MAPK abrogated hnRNP A0 binding to its MIP-2 (macrophage inflammatory protein 2) client mRNA and impaired MIP-2 mRNA stability and protein induction. Together these findings suggest that MK2-dependent

phosphorylation of hnRNP A0 is required for mRNA binding and stabilization.

A number of other RBPs have been identified as MK2 substrates *in vitro*, however, the functional relevance of these phosphorylation events remains elusive and awaits further investigation. For example, Bollig and colleagues identified PABP1 (Polyadenylate-binding protein 1) as a GM-CSF (Granulocyte macrophage colony-stimulating factor) ARE-binding protein, which can be efficiently phosphorylated by MK2 *in vivo* (Bollig et al., 2003). Whether this phosphorylation takes place *in vivo* and what influence it might have on GM-CSF mRNA stability or translation remains unclear.

Although, defects in RBPs have been associated with a large number of diseases, our current knowledge is largely still restricted to canonical RNA binding domains and target sequences (Lukong et al., 2008; Cooper et al., 2009; Darnell, 2010). However, major progress is currently being made in our understanding of RBP biology, similar to the extensive achievements concerning the role of microRNAs (miRs) in the posttranscriptional regulation of target mRNAs. Considerable accomplishments in this field were obtained from studies devoted to the systematic discovery of structural elements governing stability of mammalian mRNAs, the generation of an atlas of mammalian RBPs and the identification of target RNAs via high-throughput sequencing of cross-linked RNPs after immunoprecipitation (Hafner et al., 2010; Zhang and Darnell, 2011; Castello et al., 2012; Goodarzi et al., 2012).

MicroRNA-MEDIATED REGULATION OF THE DNA DAMAGE RESPONSE

In addition and complementary to regulation of mRNA stability and translation by RBPs, posttranscriptional control is potently exerted by miRs. These recently discovered, yet ubiquitous molecules, 18–24 nucleotides in length, regulate the stability and/or translation of their target mRNAs by forming imperfect Watson-Crick base pairs within the 3' UTR. By virtue of this interaction, the microRNA recruits a protein complex referred to as miRISC (miRNA-induced silencing complex) that exerts translational repression by a mechanism that is not yet fully understood. Recently, reported data strongly suggests that destabilization of target mRNAs, instead of translational repression, is the predominant mechanism for reduced protein output (Guo et al., 2010). The minimal protein components of miRISC required for microRNA-mediated this repression are Argonaute (AGO; principally AGO2 in mammals and AGO1 in flies) and TNRC6 (trinucleotide repeat containing 6)/GW182 (glycine-tryptophan protein of 182 kDa) (Guo et al., 2010). One, mechanism of microRNA function that has been proposed is the sequestration of their target mRNAs in sub-cellular compartments that prevent their access to the protein synthesis machinery (Cannell et al., 2008). Two, such compartments implicated in microRNA control are SGs and P-bodies (PBs), both related structures acting as sites of triage for repressed mRNA molecules (Cannell et al., 2008; Buchan and Parker, 2009). The notion that SGs may play an important role for the DDR arises from a study by Pothof et al., who showed that UV-induced DNA damage caused a transient localization of AGO2 to SGs and that cells

depleted of AGO2 are hypersensitive to UV-irradiation (Pothof et al., 2009). Furthermore, Zeng et al. demonstrated that MK2 can efficiently phosphorylate AGO2 on Ser-387 and this reaction was induced in HEK293T cells over-expressing AGO2 after treatment with sodium arsenite (Zeng et al., 2008), a known activator of the p38MAPK/MK2 pathway. Besides, examining immortalized human non-small cell lung carcinoma cells (NCI-H1299), the group showed that mutation of Ser-387 to alanine or pharmacological inhibition of p38MAPK reduced arsenite-induced AGO2 recruitment into PBs. This points to a potential role for MK2 signaling in the formation of SGs and PBs.

In addition to the global regulation of the DDR by AGO2, specific miRNAs have been shown to be vitally important for cells to mount a functional DDR. The first example found were the miRNAs of the miR-34 family (miR-34a, miR-34b, and miR-34c), which were simultaneously identified as p53 transcriptional targets by several groups (Chang et al., 2007; Corney et al., 2007; He et al., 2007; Tarasov et al., 2007). These miRNAs appear to act as critical regulators of the DDR by repressing target mRNAs that regulate the cell cycle and apoptosis. Concretely, data presented by Raver-Shapira et al. indicates that inhibition of miR-34a, the most pro-apoptotic member of the miR-34 family, prevented etoposide-induced cell death to the same extent as p53 depletion, suggesting that miR-34a is a potent mediator of p53-mediated apoptosis in this context (Raver-Shapira et al., 2007). The ability of miR-34a to induce apoptosis may be attributable to its ability to repress the anti-apoptotic protein BCL-2 via an interaction in the 3' UTR of BCL-1 mRNA (Bommer et al., 2007). However, Yamakuchi et al. showed that miR-34a represses SIRT1 through its 3' UTR and that over-expression of SIRT1 rescued miR-34a-induced apoptosis (Yamakuchi et al., 2008), suggesting that SIRT1 is a functionally important target in that system. In contrast to miR-34a, miR-34b/c do not seem to regulate cell death. Rather, these two highly homologous miRNAs inhibit cell cycle progression in response to DNA damage primarily by repressing the proto-oncogene C-MYC in both a p53-dependent and -independent manner (Cannell and Bushell, 2010; Cannell et al., 2010).

Since the initial finding of miR-34, several other miRNAs regulating events both proximal and distal to the initial DNA lesion, have been implicated in the DDR. WIP1 (wild-type p53-induced phosphatase 1), a key phosphatase targeting critical DDR components, such as p53, ATM, and H2AX for dephosphorylation, is also the target of a miRNA (Takekawa et al., 2000; Lu et al., 2005; Shreeram et al., 2006). Specifically, the experiments performed by Zhang et al. (2010) revealed that miR-16, a tumor suppressor miRNA frequently found to be deleted in chronic lymphocytic leukemia (CLL), inhibits WIP1 translation (Calin et al., 2002, 2004; Zhang et al., 2010). According to the authors, WIP1 mRNA levels rapidly increase following DNA damage, while WIP1 protein fails to accumulate. Further, they went on to show that miR-16 levels augment rapidly in response to neocarzinostatin, consequently prevent WIP1 protein accumulation and thus allowing ATM phosphorylation to be maintained. At later stages, likely when DNA repair is complete, miR-16 levels decrease, WIP1 protein accumulates again and ATM is dephosphorylated (Zhang et al., 2010). These observations are particularly pertinent in

the context of p53 signaling: as well as transcriptionally regulating miR-34, p53 also controls the maturation of certain miRNAs including miR-16 in a posttranscriptional manner (Suzuki et al., 2009). At birth, miRNAs are long primary transcripts termed pri-miRs and are processed in the nucleus by an enzyme called Drosha to become a pre-microRNA (60–70 nucleotides in length). This pre-microRNA is further exported to the cytoplasm and subjected to the RNase III enzyme Dicer for final processing (18–24 nucleotides). Interestingly, Suzuki et al. demonstrated that p53 forms a complex with Drosha by virtue of an interaction with the DEAD-box RNA helicase p68 (a.k.a DDX5) to augment conversion of pri-miR-16 (amongst others) in a DNA damage-dependent manner (Suzuki et al., 2009). Considering the observation that WIP1 is also a p53 target gene (Fiscella et al., 1997), allows us to hypothesize on the following scenario: p53 transcriptionally induces WIP1 and posttranscriptionally induces miR-16, which limits WIP1 protein production. Upon completion of DNA repair, miR-16 levels decrease and lead to a rise in WIP1 protein and attenuation of ATM signaling. It is tempting to speculate that the association between p53 and p68/DDX5 is regulated by alternative DNA damage signaling pathways to those, which control p53-dependent transcription leading to differential temporal regulation of p53-mediated transcription and miRNA processing.

In addition to the above, downstream events in the DDR signaling cascade are also regulated by miRNAs. By generating cell lines deficient for miR-21, Wang et al. demonstrated that CDC25A is regulated by this miRNA via its 3' UTR (Wang et al., 2009). The analyses of miR-21 deficient RKO colon cancer cells disclosed increased mitotic entry in response to IR in comparison to their wild-type counterparts. This phenomenon was largely blunted by CDC25A depletion, suggesting that miR-21 regulates a DNA damage induced G₂/M checkpoint by repressing CDC25A (Wang et al., 2009). It is therefore possible that DNA damage imposes a “double-hit” inhibition on CDC25A function by restraining its translation through miR-21 and promoting its degradation through Chk1/Chk2/MK2 signaling (Reinhardt and Yaffe, 2009). However, it remains enigmatic who are the key players promoting induction of miR-21 in response to DNA damage and whether this executed at the transcriptional or posttranscriptional level.

Very recently, Gorospe and colleagues have uncovered some of the mechanisms mediating miR-519-dependent regulation of the DDR (Abdelmohsen et al., 2012). It was previously known that miR-519 inhibits cell proliferation. This group now identified two prominent subsets of miR-519-regulated mRNAs. First, miR-519 targets mRNAs encoding the DNA maintenance proteins DUT1, EXO1, RPA2, and POLE4 to repress their expression ultimately resulting in increased DNA damage and upregulation of CDKN1A^{P21}. The second group of target mRNAs encoded proteins involved in calcium homeostasis, such as, ATP2C1 and ORAI1. Downregulation of these mRNAs raised cytosolic calcium levels, further increasing p21 levels. Together these alterations produced an autophagic phenotype in various cell lines.

Although, the majority of studies regarding non-coding RNA has focused on the function of miRNAs, a plethora of non-coding transcripts still awaits to be analyzed for their role in

the DDR [for a detailed review on non-coding RNA in diverse human diseases see (Esteller, 2011)]. Recently, more than 1000 large intergenic noncoding RNAs (lincRNAs) have been reported (Khalil et al., 2009). These RNAs are evolutionarily conserved in mammalian genomes and thus presumably function in diverse biological processes (Khalil et al., 2009). Interestingly, lincRNA-p21 (located near the *CDKN1A* gene encoding the p21 protein) is transcriptionally regulated by p53 and was also shown to interact with hnRNP-K, namely by conveying hnRNP-K to the promoter region of p53 target genes, which in turn become transcriptionally repressed (Huarte et al., 2010). This lincRNA-p21:hnRNP-K interaction was observed to be required for proper genomic localization of hnRNP-K at repressed genes and regulation of p53-mediated apoptosis (Huarte et al., 2010).

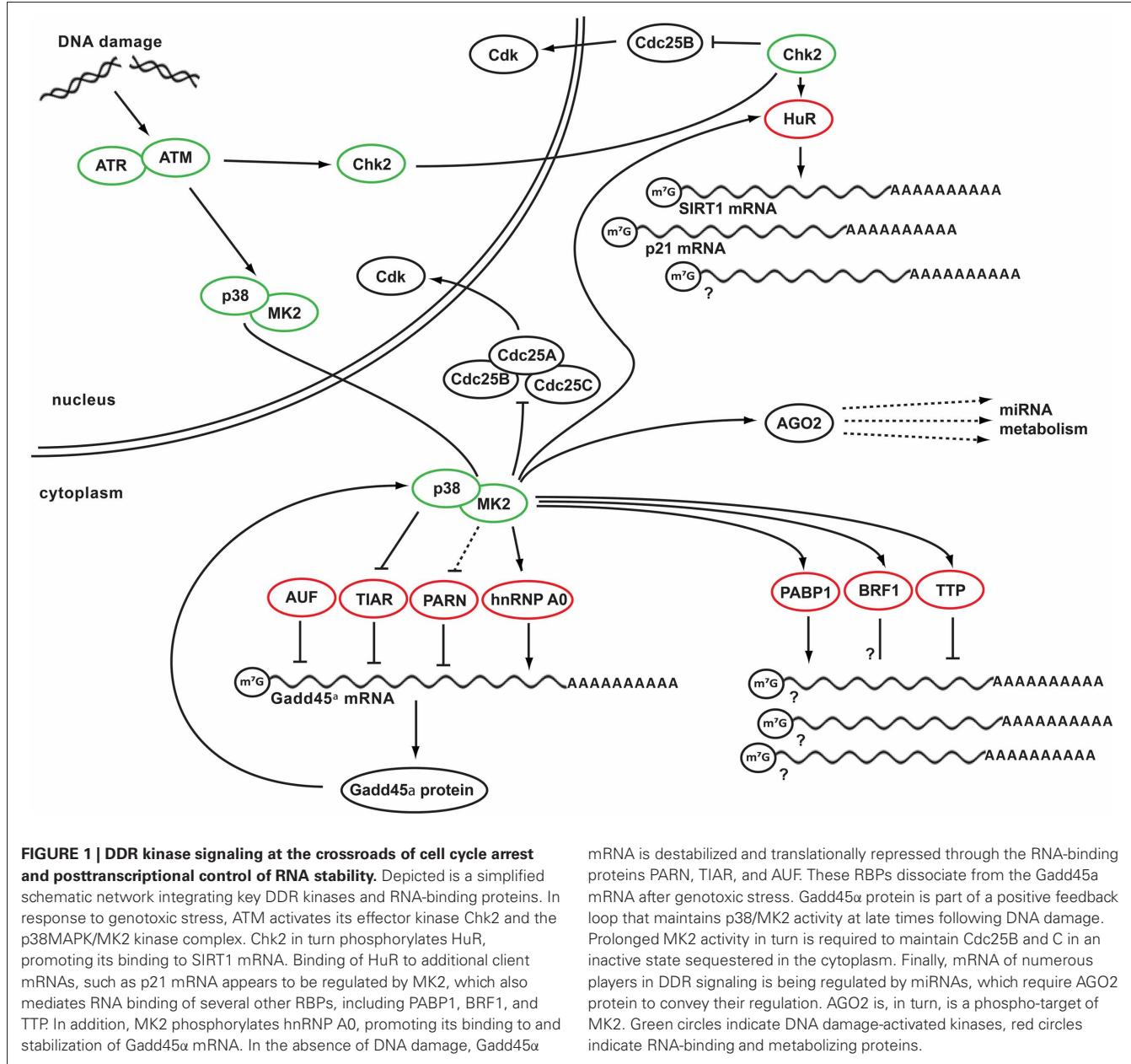
More recently, Wei and colleagues elegantly illustrated that so-called DSB-induced small RNAs (diRNAs) are transcribed from sense and antisense strands at, or close to the DSB sites in *Arabidopsis* and human cells (Wei et al., 2012). In *Arabidopsis*, the biogenesis of diRNAs required ATR, RNA Pol IV, and Dicer-like proteins. Mutations in these proteins as well as in Pol V prevented efficient DSB repair (Wei et al., 2012). Subsequently, the authors provided evidence that diRNAs are recruited byAGO2 to establish DSB repair in *Arabidopsis*. Furthermore, depletion of Dicer or AGO2 in human cells led to a similar decrease in DSB repair efficiency. The authors propose diRNAs to serve as guiding molecules directing chromatin modifications or the recruitment of protein complexes to DSB sites in order to ultimately facilitate DSB repair (Wei et al., 2012).

GADD45 α IS POSTTRANSCRIPTIONALLY REGULATED IN RESPONSE TO DNA DAMAGE

In addition to p21^{Cip1/WAF} mRNA, which has been demonstrated to be posttranscriptionally stabilized after DNA damage, Fornace and colleagues identified Gadd45 α mRNA as posttranscriptionally stabilized in response to genotoxic stress (Jackman et al., 1994) (Figure 1). Gadd45 α is part of a family of genes consisting of Gadd45 α , Gadd45 β , and Gadd45 γ that is widely expressed in mammalian cells following different stress stimuli. Gadd45 α is induced following hypoxia, IR, oxidants, UV, and growth factor withdrawal (Zhan, 2005). Gadd45 α has been mechanistically linked to numerous cellular processes, including apoptosis, cell cycle arrest, nucleotide excision repair and repair-mediated DNA demethylation, maintenance of genomic stability and signaling through the p38MAPK, and JNK kinase pathways (Hollander et al., 1999; Wang et al., 1999; Smith et al., 2000; Amundson et al., 2002; Hildesheim et al., 2002; Barreto et al., 2007). Gadd45 α expression is rapidly induced after genotoxic stress. This transcriptional activation has initially been thought to be primarily induced by p53 (Kastan et al., 1992). In fact, p53 was the first transcription factor reported to induce Gadd45 α transcription and, at least in response to IR, Gadd45 α transcription strictly depends on p53 (Kastan et al., 1992). However, it is now clear that additional transcription factors, including WT1, Oct1, NF-YA, FoxO3a, Egr-1, and C/EBP α are also capable of inducing Gadd45 α transcription, even in the absence of p53 (Constance et al., 1996; Zhan et al., 1998; Jin et al., 2001; Takahashi et al., 2001; Tran et al., 2002; Hirose et al., 2003; Thyss et al., 2005). For example, we recently

showed that Gadd45 α was induced in p53-deficient murine embryonic fibroblasts (MEFs) following treatment with doxorubicin (Jiang et al., 2009). In resting cells, Gadd45 α transcription appears to be repressed through c-Myc and a repressive complex consisting of ZBRK1 and BRCA1 (Marhin et al., 1997; Amundson et al., 1998; Bush et al., 1998; Zheng et al., 2000; Tan et al., 2004). Interestingly, c-Myc itself is translationally repressed through miR-34c via a highly conserved target-site within the 3' UTR in response to etoposide-induced DNA damage. While miR-34c can be induced by p53 following genotoxic stress, Cannell et al. (2010) showed that miR-34c expression in p53-deficient cells depends on the p38MAPK/MK2 signaling complex (Cannell et al., 2010). In addition to this elaborate network of transcriptional control, Fornace and colleagues reported as early as 1994 that Gadd45 α mRNA is posttranscriptionally stabilized in response to UV or MMS exposure (Jackman et al., 1994). However, the molecular details of this posttranscriptional regulation remained largely obscure. These posttranscriptional regulatory mechanisms might impact on Gadd45 α mRNA molecules at different steps of their maturation, from their *de novo* synthesis as pre-mRNA until the eventual degradation or translation. These steps include pre-mRNA splicing and maturation (3' polyadenylation, 5' capping), followed by mRNA export to the cytoplasm, sub-cytoplasmic transport, escape from ribonucleolytic cleavage and translation (Mitchell and Tollervey, 2000; Orphanides and Reinberg, 2002; Moore, 2005). Recent studies from Gorospe and co-workers have identified the RBPs AUF1 and TIAR as critical posttranscriptional regulators of Gadd45 α mRNA (Lal et al., 2006). Both proteins were found to form RNP complexes through a direct interaction with the 3' UTR of the Gadd45 α mRNA in resting cells. However, when cells were exposed to UV or MMS these RNP complexes rapidly dissociated, which correlated with a substantial increase in Gadd45 α mRNA stability and enhanced association of Gadd45 α mRNA with actively translating ribosomes and increased Gadd45 α protein accumulation (Lal et al., 2006). When Lal et al. examined the molecular mechanisms of Gadd45 α repression in resting cells, they found AUF1 to render Gadd45 α mRNA unstable while TIAR prevented the association of Gadd45 α mRNA with translating polyribosomes. Thus, the combined effect of AUF1 and TIAR is a potent repression of Gadd45 α biosynthesis through AUF1-mediated mRNA destabilization and TIAR-dependent translational suppression at resting state. The genotoxic stress-induced dissociation of AUF1 and TIAR from the Gadd45 α mRNA represents a mechanism of post-transcriptional derepression resulting in mRNA stabilization and enhanced translation in response to DNA damage. Both of these posttranscriptional regulatory steps were found to be essential for proper induction of Gadd45 α protein levels following DNA damage (Lal et al., 2006).

The report by Lal et al. implicated AUF1 and TIAR as RBPs that are critical for the posttranscriptional de-repression of Gadd45 α mRNA. However, it remained unclear which molecular mechanisms underlie the DNA damage-induced dissociation of these RBPs from the Gadd45 α mRNA. A plausible explanation might be DNA damage-dependent phosphorylation events. Indeed, AUF1 was reported to be a phospho-protein and GSK3 β and PKA were subsequently identified as kinases capable of AUF1



phosphorylation *in vivo* (Zhang et al., 1993; Wilson et al., 2003). Nonetheless, whether these phosphorylations occur *in vivo* following genotoxic stress persists to be elusive.

We have recently identified the p38MAPK/MK2 pathway as a critical regulator of RBPs that mediate posttranscriptional stabilization of Gadd45 α mRNA in response to genotoxic stress (Reinhardt et al., 2010). In analyzing the molecular details of MK2 function in response to DNA damage, we found that MK2 knockdown prevented the accumulation of Gadd45 α mRNA and protein in response to adriamycin. We identified the known MK2 substrate hnRNP A0 as a novel Gadd45 α mRNA-binding protein (Reinhardt et al., 2010). MK2-mediated phosphorylation of hnRNP A0 on Ser-84 following DNA damage was required for the formation of hnRNP A0:Gadd45 α mRNA RNP complexes and

overexpression of a non-phosphorylatable hnRNP A0 on Ser-84 to Ala mutant prevented Gadd45 α mRNA and protein accumulation in response to adriamycin (Reinhardt et al., 2010). These data suggest that MK2-dependent phosphorylation of hnRNP A0 is critical for the formation of hnRNP A0:Gadd45 α mRNA RNP complexes, which in turn appears to be essential for the post-transcriptional stabilization of Gadd45 α mRNA. In addition, we found that MK2 phosphorylates Poly-(A) ribonuclease (PARN) on Ser-557 in response to adriamycin (Reinhardt et al., 2010). Two major pathways of mRNA degradation exist in eukaryotes. In both cases, shortening of the poly(A) tail is the first, time-limiting, step. Three distinct protein complexes—Pan2/Pan3, or PAN complex; PARN; and the Ccr4/Pop2 complex—govern this deadenylation. After deadenylation, degradation occurs in 3'-5'

direction through the RNase-containing exosome complex. In an independent pathway, deadenylation is followed by removal of the 7-methyl-guanosine cap of mRNAs and then proceeds in the 5'-3' direction. The mechanisms of mRNA turnover have been reviewed recently (Meyer et al., 2004). We found PARN phosphorylation on Ser-557 to be critical for prolonged Gadd45 α mRNA and protein expression after adriamycin (Reinhardt et al., 2010). However, the molecular details of this apparent inhibition of Gadd45 α mRNA degradation remain somewhat unclear. Despite our best efforts, we failed to observe any changes in PARN activity or RNA binding affinity following MK2-mediated phosphorylation on Ser-557 (Schmedding, Reinhardt, Yaffe unpublished). In addition to these MK2-mediated posttranscriptional mechanisms of Gadd45 α mRNA stabilization, we confirmed that TIAR dissociates from the Gadd45 α mRNA in response to genotoxic stress (Reinhardt et al., 2010). Furthermore, we could show that p38MAPK directly phosphorylates TIAR after adriamycin exposure, both *in vitro* and *in vivo* [(Reinhardt et al., 2010) and Morandell, Reinhardt, Yaffe unpublished]. Pretreatment of cells with the p38 α/β -specific inhibitor SB203580 completely prevented the adriamycin-mediated dissociation of TIAR:Gadd45 α mRNA RNP complexes. Thus, we have identified three novel mechanisms of posttranscriptional Gadd45 α mRNA control. We identified hnRNP A0 as a critical MK2-dependent posttranscriptional inducer of Gadd45 α mRNA. In addition to AUF and TIAR, which have been described as posttranscriptional repressors of Gadd45 α mRNA, we have identified PARN as a further molecule that appears to be involved in Gadd45 α mRNA repression at resting state. Lastly, we could show that the DNA damage-induced dissociation of the TIAR:Gadd45 α mRNA RNP complex depends on p38MAPK-mediated TIAR phosphorylation.

In additional experiments we could confirm data provided by Bulavin et al. showing that Gadd45 α interacts with p38MAPK (Bulavin et al., 2003). Bulavin et al. further showed that Gadd45 α is critical for H-ras^{V12}-induced activation of p38MAPK. We made a similar observation in response to adriamycin-invoked genotoxic stress. RNAi-mediated knockdown of Gadd45 α prevented the prolonged phosphorylation and activation of MK2, likely through a lack of p38MAPK activity. MK2 remained active in control cells for at least 30 h. However, MK2 activity dropped precipitously after ~24 h in Gadd45 α -depleted cells. These data suggest that the initial activation of MK2 after genotoxic stress does not depend on Gadd45 α , but subsequent p38MAPK/MK2-dependent stabilization of Gadd45 α , through phosphorylation of TIAR, PARN, and hnRNP A0, becomes essential for maintaining MK2 activity at late times. Further experiments showed that particularly this late MK2 activity was critical to maintain checkpoint control after genotoxic stress invoked by doxorubicin through a mechanism involving Cdc25B/C inactivation. Members of the Cdc25 family of dual-specificity phosphatases are phosphorylated by the checkpoint effector kinases Chk1 and MK2 in response to DNA damage. We and others previously showed that the cell cycle arresting checkpoint function of MK2 is mediated through MK2-dependent Cdc25B/C phosphorylation and subsequent cytoplasmic sequestration (Lopez-Aviles et al., 2005; Manke et al., 2005; Reinhardt et al., 2007).

We note that MK2 and its activating kinase p38MAPK form a tight nuclear complex in resting cells (Ben-Levy et al., 1995,

1998; ter Haar et al., 2007). MK2 contains a nuclear localization signal (NLS) and a nuclear export signal (NES) located at the C-terminus. At resting state, the NES is masked by a direct intramolecular interaction (ter Haar et al., 2007). Following p38-mediated activating phosphorylation of MK2 on Thr-334, this interaction is relieved and the NES becomes exposed, resulting in cytoplasmic translocation of the p38MAPK/MK2 complex (Ben-Levy et al., 1995, 1998; ter Haar et al., 2007). We could show that MK2 rapidly leaves the nucleus in response to DNA damage via a Crm1-dependent nuclear export mechanism (Reinhardt et al., 2010). Thus, we hypothesized that late cytoplasmic MK2 activity might be required to maintain Cdc25B/C sequestered in the cytoplasm in the context of active cell cycle checkpoints. We have hence used live cell imaging to follow the subcellular distribution of Cdc25B and C after genotoxic stress in control cells or cells that were depleted of either Chk1 or MK2. Cytoplasmic accumulation of GFP-tagged Cdc25B/C was used as a readout for active checkpoint signaling. These experiments revealed that adriamycin exposure induces a robust cell cycle checkpoint in control cells that is relieved after ~30 h and is followed by a cytologically normal mitotic cell division. Cdc25B/C was maintained in the cytoplasm until cells entered mitosis. In contrast, Chk1 depletion resulted in premature nuclear re-entry of Cdc25B/C after ~15 h, followed by catastrophic mitotic cell division resulting in apoptosis. We observed a similar phenotype in MK2-depleted cells. However, Cdc25B/C nuclear re-entry did not occur until ~23 h following doxorubicin. Intriguingly in this regard is the observation that this time corresponds perfectly to the time when MK2 activity returned to baseline levels in Gadd45 α -depleted cells that were treated with doxorubicin. These data strongly suggest that the positive feedback loop involving MK2-dependent stabilization of Gadd45 α , and Gadd45 α -dependent maintenance of MK2 activity, are essential for prolonged cell cycle arrest through cytoplasmic Cdc25B/C sequestration in response to adriamycin. Together, these data suggest that a feed forward loop consisting of p38, MK2, and Gadd45 α is critical to provide time to recover from adriamycin-induced genotoxic insults before entering the next mitotic cell division.

CONCLUDING REMARKS

Posttranscriptional control of gene expression has recently moved into the focus of scientists working in various areas of life sciences. This is owed to the discovery of miRNA-mediated gene silencing mechanisms and the uncovering and characterization of a number of RBPs that are involved in the stabilization and translatability of mRNAs. The DDR network has classically been regarded as consisting of a fast-acting kinase signaling branch, leading to the rapid inactivation of Cdk-cyclin complexes and a delayed transcriptional response, resulting in the transactivation of genes encoding for Cdk inhibitors, such as p21. As a consequence of numerous recent discoveries, a clearer picture is emerging stressing the molecular mechanisms involved in posttranscriptional control of gene expression and expanding the complex DDR signaling network with a third layer. These recent reports strongly suggest that cells employ complex regulatory circuits impacting on transcript stability and translatability in response to genotoxic stress. The major challenges in this emerging area of research in the field of DNA damage signaling

are the identification of transcripts that are posttranscriptionally regulated and the identification and functional characterization of proteins that mediate this posttranscriptional control. New technologies, such as, genome-wide RNAi screening and next generation sequencing of cell lines and primary tumor material will promote the identification and functional characterization of non-coding RNAs, RBP, and regulatory RNA sequences involved in the initiation, maintenance and termination of DDR signaling in human tissue.

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Highlighting the DNA damage response with ultrashort laser pulses in the near infrared and kinetic modeling

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Our understanding of the mechanisms governing the response to DNA damage in higher eucaryotes crucially depends on our ability to dissect the temporal and spatial organization of the cellular machinery responsible for maintaining genomic integrity. To achieve this goal, we need experimental tools to inflict DNA lesions with high spatial precision at pre-defined locations, and to visualize the ensuing reactions with adequate temporal resolution. Near-infrared femtosecond laser pulses focused through high-aperture objective lenses of advanced scanning microscopes offer the advantage of inducing DNA damage in a 3D-confined volume of subnuclear dimensions. This high spatial resolution results from the highly non-linear nature of the excitation process. Here we review recent progress based on the increasing availability of widely tunable and user-friendly technology of ultrafast lasers in the near infrared. We present a critical evaluation of this approach for DNA microdamage as compared to the currently prevalent use of UV or VIS laser irradiation, the latter in combination with photosensitizers. Current and future applications in the field of DNA repair and DNA-damage dependent chromatin dynamics are outlined. Finally, we discuss the requirement for proper simulation and quantitative modeling. We focus in particular on approaches to measure the effect of DNA damage on the mobility of nuclear proteins and consider the pros and cons of frequently used analysis models for FRAP and photoactivation and their applicability to non-linear photoperturbation experiments.

Keywords: non-linear optics, fluorescence, microirradiation, DNA strand break

INTRODUCTION

The DNA damage response plays a crucial role in oncogenesis (Bartek et al., 2007, 2012). Consequently, cancer biologists have a strong interest in experimental methods that address the spatiotemporal dynamics of the complex chain of cellular events triggered by DNA lesions. In recent years, microirradiation with focused laser beams has emerged as a useful tool to introduce DNA damage in live cells and to study the ensuing cellular responses via fluorescence imaging. In the first part of this Review, we will recapitulate the pros and cons of the most widespread microirradiation methods and point at new developments involving the use of pulsed near infrared lasers.

In eucaryotes, all reactions to DNA damage occur within chromatin. The local chromatin environment affects the susceptibility to genotoxic agents, influences the choice of DNA repair pathway and the efficiency of the repair reaction (Ziv et al., 2006; Fernandez-Capetillo and Murga, 2008; Jakob et al., 2011; Xu and Price, 2011). Despite successful restoration of the DNA sequence, long-lasting and heritable marks that persist at the level of chromatin structure may contribute to cellular transformation and cancer at a later stage (Lukas et al., 2011). To understand the biological consequences of genotoxic stress it is therefore essential to integrate the role of chromatin into our picture of the DNA

damage response. Methods for visualizing chromatin rearrangements induced by DNA damage in living cells at high spatial and temporal resolution are of fundamental importance to achieve this goal. We will present imaging techniques suited to this purpose in the second part of this article focusing on combinations of laser microirradiation and fluorescence photoperturbation. Finally, to achieve an accurate quantitative description of how chromatin dynamics is affected by DNA lesions kinetic data need to be correctly interpreted. We will conclude this Review discussing current modeling approaches and their appropriateness for quantifying alterations of protein mobilities in microirradiated nuclei.

LASER MICROIRRADIATION METHODS FOR THE INDUCTION OF LOCALIZED DNA DAMAGE

Among the available methods to expose cells to DNA damage like treatment with genotoxic chemicals or diffused irradiation, focused laser beams offer the fundamental advantage of free choice of the target area. With respect to irradiation with heavy ions or alpha particles which can also be employed for this purpose (Heiss et al., 2006), laser sources are easier to handle and to integrate into confocal microscopes for sample observation. Using high aperture objective lenses, the microbeam can be

directed to any subcellular or subnuclear region of interest. UV lasers have been used for this purpose since the late 60's and were applied to the study of chromosome structure and of the repair of UV-photolesions in the nucleus (Berns et al., 1969; Berns, 1978; Cremer et al., 1981). In this wavelength region damage to DNA occurs mainly via direct linear absorption. Selective induction of UV-photoproducts, i.e., cyclobutane pyrimidine dimers (CPDs) was reported at 266 nm, a wavelength well-matching the absorption maximum of DNA bases at 260 nm (Voet et al., 1963). At longer wavelengths (340–500 nm), side reactions are observed due to the production of reactive oxygen species in the aqueous cellular environment which also contains endogenous sensitizers. The outcome are (unwanted) oxidative base modifications such as 8-oxo-guanine and DNA strand breaks (Kielbassa et al., 1997). The main microirradiation studies exploiting linear absorption in the UV/VIS region to induce localized DNA damage are summarized in **Table 1**.

In the presence of exogenous photosensitizers such as the base analogue bromodeoxyuridine (BrdU) or the intercalating dye Hoechst, exposure of cells to UVA illumination leads to single and double strand breaks (Limoli and Ward, 1993). Rougakou et al. exploited this finding to introduce DNA strand breaks in nuclei irradiated with a UVA laser at a wavelength of 390 nm (Rougakou et al., 1999). Since then, the combination of photosensitization and UVA has become very popular because it can be easily performed using a GaN-based UV laser diode at a wavelength of

405 nm which is frequently provided with commercial confocal microscopes. However, at this longer wavelength and only in presence of Hoechst 33342 a residual production of CPD has been reported (see **Table 2**). The combination of this sensitizer with irradiation at 405 nm seems to trigger an unusual and yet poorly characterized response (Dinant et al., 2007). In general, photosensitizers may have undesired effects on chromatin structure and cellular metabolism, not to mention the fact that they will mediate additional damage throughout the imaging procedure performed with visible light (Solarczyk et al., 2012).

In sum, methods based on linear absorption achieve optimal damage specificity with high efficiency only in the UVC region around 260 nm, leading to almost exclusive induction of UV-photoproducts. This technique requires specialized optics with high UV transmittance. In addition, Solarczyk et al. demonstrated selective induction of DNA strand breaks at 488 nm at power levels normally used in confocal imaging (Solarczyk et al., 2012). Independently of the wavelength used, linear absorption occurs throughout the entire irradiation path. Although the photon flux above and below the focal plane is much lower than in the focus of the objective lens, this may lead to substantial damage and, in flat cultured cells, cause harm to the nuclear membrane.

To achieve confinement of the photomanipulated volume in three dimensions, multiphoton (non-linear) excitation is the tool of choice. First described in theory by Maria Göppert-Mayer in 1931 and demonstrated experimentally by Kaiser and Garrett

Table 1 | Laser microirradiation methods to induce DNA damage predominantly via linear absorption.

Reference	Laser type	Wavelength (nm)	Type of DNA damage detected
Dinant et al., 2007	Diode pumped solid state laser (2 mW, 7.8 kHz)	266	CPD (IF) 6-4PP (IF) no DSB (γ H2AX-IF, TUNEL)
Kong et al., 2009	Nitrogen laser (4 ns; 6 Hz, 0.04 μ J/pulse)	337	CPD (IF) 4-6PP (IF) 8-oxoG (IF) DSB (Ku70 recruitment)
Lan et al., 2004	Not reported	365	DSB (γ H2AX-IF) 8-oxoG (OGG1 recruitment)
Solarczyk et al., 2012	CW Ar ⁺ -ion laser (1.7 mW)	488	DSB (γ H2AX-IF) DSB (phospho-ATM, RPA, XRCC1, Lig III, PCNA recruitment)

The induced lesions were characterized via immunofluorescence (IF), terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and recruitment of DNA repair factors, as indicated. CPD, cyclobutane pyrimidine dimers; 6-4PP, 6-4 photoproduct; DSB, DNA strand break; 8-oxoG, 8-oxo-guanine; γ H2AX, phosphorylated histone H2AX.

Table 2 | Comparison of laser microirradiation methods to induce DNA damage in the presence of photosensitizers.

Reference	Sensitizer/laser type	Wavelength (nm)	Type of damage detected
Lukas et al., 2003	BrdU/nitrogen laser (30 Hz)	337	DSB (γ H2AX-IF)
Kong et al., 2009	BrdU/nitrogen laser (4 ns; 6 Hz)	337	DSB (γ H2AX-IF)
Rougakou et al., 1999	Hoechst 33258/laser type not reported	390	DSB (γ H2AX-IF)
Paull et al., 2000	Hoechst 33258/laser type not reported	390	DSB (γ H2AX-IF) 8-oxoG (OGG1)
Kong et al., 2009	BrdU/laser diode (cw)	405	DSB (γ H2AX-IF)
Dinant et al., 2007	Hoechst 33342/laser diode (cw)	405	DSB (γ H2AX-IF, TUNEL) CPD (IF)
Lan et al., 2005	Hoechst 33342/laser diode (cw)	405	DSB (γ H2AX-IF) 8-oxoG (OGG1)
Zarebski et al., 2009	Ethidium bromide/Ar ⁺ -ion laser (cw)	514	DSB (γ H2AX-IF) 8-oxoG (IF) XRCC1 (IF) HP1 β , HP1 γ (IF) HP1 β , HP1 α recruitment

The induced lesions were characterized via immunofluorescence (IF), terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and recruitment of DNA repair factors, as indicated. CPD, cyclobutane pyrimidine dimer; DSB, DNA strand break; 8-oxoG, 8-oxo-guanine; γ H2AX, phosphorylated histone H2AX.

in 1961, this process relies on the simultaneous absorption of multiple photons at very high photon densities, as they are present within the focus of the objective lens (Göppert-Mayer, 1931; Kaiser and Garrett, 1961). At high numerical apertures the affected volume is restricted to a few femtoliters. The high intensities (GW/cm^2) required for these transitions are delivered via ultrashort pulses (ps to fs) thus limiting the average laser power to levels compatible with cell viability. For non-linear excitation, the sum of the energy of the incoming photons has to match the definite energy gap between two electronic states. For DNA bases, the maximum of linear absorption lies at 260 nm and excitation at this wavelength leads to the formation of UV-photoproducts, as mentioned above. Hence, the same type of lesion can be generated by irradiating cell nuclei with femtosecond pulses at a wavelength of 780 nm, corresponding to the simultaneous absorption of three near-infrared photons (Meldrum et al., 2003; Trautlein et al., 2008), or in the visible range ($\sim 500 \text{ nm}$) via a two-photon process (Daddysman and Fecko, 2011). The relative probabilities of linear and non-linear absorption processes depend strongly on the intensities of the light field applied. Generation of UV-photoproducts via two-photon absorption at the relative moderate intensities used for imaging with continuous wave lasers in the visible range is very inefficient and can thus be neglected.

An additional mechanism that has been proposed to contribute to DNA damage by ultrashort NIR pulses is low-density plasma formation (Kong et al., 2009; Botchway et al., 2010). The high photon density and the extremely strong peak electric field of the laser pulse within the small focal volume may lead to photoionization events. In the presence of water and reactive biomolecules solvated electrons and radical intermediates are formed. This low-density plasma has been observed in pure water after exposure to femtosecond laser pulses over a broad range of conditions (Vogel et al., 2005). Its effects on DNA compare to that of ionizing radiation and include the generation of single and double strand breaks and chemical base modifications.

Thermal heating arising from laser irradiation is expected to play only a minor role as a source of DNA damage. According to calculations of Schönle and Hell irradiation of water for 1 s at a wavelength of 850 nm with 100 mW average power ($NA = 1.2$) causes a temperature rise of 0.2 K due to linear absorption (Schönle and Hell, 1998). Commonly used average powers range far below these conditions. In addition to linear absorption, the high peak power of the ultrashort laser pulses leads to collisions between free electrons and atoms and therefore to a local rise of the temperature. Although this heating can denature biomolecules, the chemical reactions triggered by free electrons strongly dominate the process of DNA damage (Vogel et al., 2005).

So far, most studies have observed a complex mixture of products after non-linear excitation of nuclear DNA (see **Table 3**). A direct comparison of the results is difficult because the exact irradiation parameters (wavelength, pulse duration, peak power and diameter of the focal spot) are not always fully specified. Furthermore, damage detection methods may vary. Not all types of lesions can be detected directly at the level of the DNA structure. So far, specific and robust antibodies are available for UV-photoproducts only. Methods for the direct detection of base

modifications in cells *in situ* are not as well-established. DNA strand breaks have been monitored via TUNEL and Comet assays (Dinant et al., 2007; Harper et al., 2008), but more frequently via antibodies recognizing the phosphorylated form of the histone H2AX (Rogakou et al., 1999), although the latter may also occur subsequent to UV-damage (Halicka et al., 2005). The presence of a defined type of lesion is often inferred from the binding of a fluorescently labeled repair factor in live-cell microscopy experiments. Given the high degree of cross-talk between DNA

Table 3 | Laser microirradiation methods to induce DNA damage via non-linear absorption.

Reference	Wavelength (nm)	Laser type	Type of damage detected
Roukos et al., 2011	355	Frequency tripled Nd:YAG laser (470 ps; 500 Hz)	DSB (γ H2AX-IF)
Daddysman and Fecko, 2011	400–525	Frequency doubled Ti:sapphire laser (210 fs; 80 MHz)	CPD (IF)
Kong et al., 2009	532	Frequency doubled Nd:YVO4 laser (12 ps; 76 Hz)	CPD (IF) 6-4PP (IF) DSB (Ku70; 53BP1 recruitment) no 8-oxoG (IF)
Meldrum et al., 2003	750	Ti:sapphire laser (120 fs; 82 MHz)	CPD (IF)
Trautlein et al., 2009	775	Frequency doubled Er:fiber laser (230 fs; 107 MHz)	CPD (IF) 6-4PP (IF) DSB (γ H2AX-IF)
Mari et al., 2006	800	Ti:sapphire laser (200 fs; 76 MHz)	DSB (γ H2AX-IF)
Kong et al., 2009	800	Ti:sapphire laser (200 fs; 76 MHz)	CPD (IF) 6-4PP (IF) DSB (Ku70; 53BP1 recruitment) no 8-oxoG (IF)
Inagaki et al., 2009	800	Ti:sapphire laser (200 fs; 76 MHz)	CPD (IF) DSB (γ H2AX-IF)
Dinant et al., 2007	800	Ti:sapphire laser (200 fs; 76 MHz)	CPD (IF) 6-4PP (IF) DSB (γ H2AX-IF)
Trautlein et al., 2009	1050	Yb:fiber laser (77 fs; 107 MHz)	no CPD (IF) no 6-4PP (IF) DSB (γ H2AX-IF)

The induced lesions were characterized via immunofluorescence (IF), and recruitment of DNA repair factors, as indicated. CPD, cyclobutane pyrimidine dimer; 6-4PP, 6-4 photoproduct; DSB, DNA strand break; 8-oxoG, 8-oxo-guanine; γ H2AX, phosphorylated histone H2AX.

repair pathways the concomitant occurrence of multiple lesions cannot be excluded by this approach. More extensive reviews comparing the outcome of laser-induced DNA microdamaging methods, including NIR irradiation, are provided in (Dinant et al., 2007; Inagaki et al., 2009; Kong et al., 2009; Nagy and Soutoglou, 2009; Botchway et al., 2010).

Mode locked Ti:sapphire laser oscillators are frequently used to induce DNA damage with NIR irradiation because these systems are well-established as excitation sources for commercial two-photon confocal microscopes. For multiphoton imaging they are usually operated at a wavelength of ~ 800 nm delivering pulses of a duration of 100–200 fs. From the studies mentioned above it has become clear that these pulses produce a mixture of lesions with strand breaks and photoproducts being detectable at similar levels. What is the perspective for improving the lesion specificity of pulsed NIR irradiation in order to fully exploit the advantages of three-dimensional confinement of the damage and low collateral damage? As we have recently shown, one way to achieve this goal is to increase the center wavelength of the pulse: at $\lambda = 1050$ nm the efficiency of formation of UV photoproducts drops drastically while that of DNA strand breaks remains unaltered. This observation let us propose an irradiation method that leads to the preferential induction of DNA strand breaks vs. UV photoproducts without the need of exogenous photosensitizers (Trautlein et al., 2009). As this work suggests, the efficiency of the different damage mechanisms may depend distinctly not only on the wavelength, but also on other pulse parameters. Along this line, Kong et al. have reported an impact of repetition rate and pulse duration on the threshold for phosphorylation of H2AX (Kong et al., 2009) while another study has shown a dependence of cell viability from pulse length at constant pulse energy (Konig et al., 1999).

In sum, non-linear excitation with ultrashort near infrared laser pulses enables to introduce highly localized DNA lesions on a sub- μm scale in the nuclei of living cells with minimum collateral photodamage. As future perspective we envision that these pulses may be tailored to become lesion-specific through a careful analysis of the influence of parameters such as laser central wavelength, pulse duration, repetition rate and peak irradiation on the type of damage.

IMAGING APPROACHES FOR VISUALIZING CHROMATIN DYNAMICS AT SITES OF LOCAL DNA DAMAGE

The ability to target only a selected small area of the nucleus via laser microirradiation is one important prerequisite for addressing the role of nuclear organization in the chromatin response to DNA damage. A reliable indicator of chromatin remodeling is histone mobility as can be measured via fluorescence photoperturbation methods. Fluorescence recovery after photobleaching (FRAP), and, to a lesser extent, photoactivation, have yielded important insights into the dynamics and binding properties of histones in intact, native chromatin (Kimura, 2005; Beaudouin et al., 2006; Wiesmeijer et al., 2008; Martin and Cardoso, 2010; Raghuram et al., 2010; Stasevich et al., 2010). Mobility changes triggered by DNA damage can be investigated easily on a global scale, by treating cells with genotoxic agents such as chemicals or ionizing radiation prior to a FRAP or photoactivation

experiment. These approaches disregard local differences in chromatin architecture, and they detect mobility changes only on the time-scale of several minutes to hours depending on the type and duration of the genotoxic treatment. To study the effect of local DNA damage on chromatin dynamics one can perform a FRAP experiment within a subnuclear region previously exposed to the DNA damaging laser. This strategy is straightforward if the fluorescently labeled protein of interest visibly binds to the damage and accrues along the laser trajectory. The photobleaching laser is then targeted to this area when the signal increase, i.e., the binding reaction in the irradiated region, has reached steady-state to avoid superimposition of the recruitment and the bleaching recovery kinetics. Using this approach, Mortusewicz et al have shown distinct mobilities of XRCC1 and PCNA at DNA damage induced at 405 nm (Mortusewicz and Leonhardt, 2007), while two other studies have investigated the biphasic dynamics of HP1- β and compared the mobilities of Cdt1, Cdt2 and PCNA at laser-irradiated sites, respectively (Ayoub et al., 2008; Roukos et al., 2011). Further reports compared the recovery kinetics of PARP1 and a catalytic mutant at DNA damage (Mortusewicz et al., 2007). The behavior of members of the MRN complex and of 53BP1 at DNA strand breaks was also investigated (Lukas et al., 2004; Bekker-Jensen et al., 2005). For proteins that do not recruit to DNA damage including most chromatin components it is possible to measure FRAP curves within a pre-irradiated, damaged area via its known coordinates. However, unintentional bleaching due to the damaging laser—in particular if wavelengths in the UV/VIS are used—will interfere with the subsequent FRAP experiment. The latter can be performed only after the signal has recovered from the first irradiation step, thus severely limiting the temporal resolution of this approach.

Fluorescence photoactivation has also been combined with laser microirradiation to study local changes of chromatin structure due to DNA strand breaks (Kruhlak et al., 2006). In this study, cells expressing a photoactivatable fusion of histone H2B (PAGFP-H2B) were irradiated with a UV laser (364 nm) either in the absence or presence of the DNA intercalator Hoechst 33342. DNA strand breaks were produced only under the latter condition, as demonstrated by the appearance of phosphorylated H2A along the laser track. Under both conditions the DNA damaging laser simultaneously activated PAGFP-H2B. Significantly, a local broadening of the fluorescence signal could be observed only in the presence of the sensitizer indicating an expansion of chromatin in the direct proximity of DNA strand breaks.

These examples highlight one basic requirement for combining DNA damage via laser microirradiation with fluorescence photoperturbation for mobility measurements: the two irradiation procedures must not influence each other, i.e., they have to either induce DNA damage or photobleach/photoactivate, respectively. In the study by Kruhlak et al., this distinction was reached by adding a photosensitizer. Recently, we have presented an alternative strategy based on the use of femtosecond laser pulses of different wavelengths (Tomas et al., 2012). In a first step, DNA strand breaks are introduced via irradiation at $\lambda = 1050$ nm in a defined subnuclear volume, as described above. Subsequently the protein to be studied, which is expressed as a photoactivatable fusion is spot-activated within the lesioned zone via two-photon

absorption at $\lambda = 775$ nm. We demonstrate that the two irradiation procedures can be performed in a single cell nucleus without mutual interference if the pulse parameters are chosen properly. Under these conditions it is possible to vary the time delay between the infliction of damage at 1050 nm and the photoactivation step at 775 nm arbitrarily. Hence, alterations in protein mobilities can be probed at different stages of the DNA damage response. The spatial precision of non-linear excitation also enables to vary the position of the photoactivation spot with respect to the damaged region. Using this method, we could show that DNA strand breaks lead to an increase in the mobility of histone H1.2 in the time range of 2 min after infliction of damage and that this change is spatially confined because distant chromatin is not affected (Tomas et al., 2012). The spatial and temporal flexibility of this assay will enable to visualize how the chromatin response to DNA damage emanates within the cell nucleus thus contributing to dissect the spatiotemporal complexity of this fundamental biological process.

MODELING CHANGES IN CHROMATIN DYNAMICS IN RESPONSE TO LOCAL DNA DAMAGE

To extract information from the complex data sets on the kinetics generated by the combination of laser microdamage and fluorescence photoperturbation it is necessary to elaborate proper modeling approaches. Their aim is to describe quantitatively how structural damage affects the interaction of DNA with chromatin proteins.

Numerous studies have undertaken quantitative analysis of FRAP and photoactivation data of nuclear proteins under undisturbed conditions (i.e., in the absence of intentional DNA damage). In general, a reaction-diffusion model is assumed to be the best mathematical description of the dynamic behavior of these proteins. According to our current understanding, nuclear proteins diffuse stochastically in all three dimensions within the nucleoplasm until they collide with binding partners with whom they undergo transient interactions. This model assigns to each protein the following characteristic parameters: the binding and release constants k_{on} and k_{off} describing binding events, and the diffusion coefficient D accounting for phases of unrestrained movement. Solving the system of differential equations required for a full reaction-diffusion model is a complex task. Therefore, different simplifying assumptions have been made in different studies, depending on the experimental setting used to generate the mobility data (FRAP, or photoactivation, size of the bleached/photoactivated area etc.). These simplifications have enabled analytical solutions of the reaction-diffusion model, exactly describing the redistribution of the fluorescence signal to equilibrium as a function of time. The basic assumptions that are employed for this type of approach are the following:

1. The system's properties remain close to unperturbed, i.e., the number and the distribution of binding sites do not change during the observation period. In addition, binding sites are assumed to be homogeneously distributed within the nucleus. The latter does not hold true for chromatin proteins because chromatin density varies between subnuclear regions (e.g., in hetero- vs. euchromatin.).

2. The photobleached/photoactivated volume has a uniform extension along the z-dimension. According to this assumption intensity changes introduced by the photobleaching/activating laser do not vary along the optical axis, and the system can be described by a two-dimensional model.
3. The finite dimension and the geometry of the cell nucleus can be neglected. This simplification applies only when the photobleached/photoactivated spot is small as compared to the nuclear volume.

Otherwise, the solution of the reaction-diffusion model has to be found via numerical modeling where changes in fluorescence are approximated consecutively for each time point for a given set of parameters. For both the analytical and the numerical procedure, the obtained solutions are optimized by testing combinations of different parameters iteratively until the simulated behavior fits the experimental data.

The power of kinetic modeling consists in its ability to make quantitative predictions about the reaction of the biological system under study. For FRAP models, this ability has been questioned, because different approaches have yielded very different results for the same or similar proteins. Therefore, a cross-validation strategy that compares different models as well as different experimental methods to generate the primary data is highly recommended (Mazza et al., 2008, 2012; Mueller et al., 2010).

For modeling changes of nuclear protein dynamics triggered by localized DNA damage yet other issues have to be considered. The main difference with respect to the undamaged situation is that at the time point of the mobility measurement the system itself may not be in an unperturbed state (see point 1 in the above list). Exposure to short laser pulses to induce DNA damage initiates a signal chain that may develop on different time scales for different types of lesions/binding sites. This amplification process is inherent to the biological response to DNA damage and leads to a fast and massive propagation of the damage signal, as best exemplified by the spreading of the phosphorylation of histone H2AX from the initial strand break to chromatin regions of the size of a few megabases (Rogakou et al., 1999; Iacovoni et al., 2010). Docking sites for proteins are thus generated in reactions secondary to proper DNA damage, as has been described in detail for many DNA repair factors, but also holds true for chromatin constituents (see e.g., Li et al., 2010; Lukas et al., 2011; Xu et al., 2012). To verify if the steady-state criterium is fulfilled it is thus very important to assess qualitatively on which time scale the mobility of the protein under investigation is influenced by DNA damage. The equilibrium approximation can be made only for observation times that are much shorter than the chromatin remodeling processes themselves.

Concerning the number and distribution of binding sites, a spatial discontinuity is implicit in the localized nature of the damage inflicted by microirradiation. Obviously, a different number of binding sites is present within the damaged region as compared to the rest of the nucleoplasm. The approximation of a uniform distribution can be made only if the area in which fluorescence redistribution is measured is much smaller than the DNA damaged one, and only for short observation times during which the

protein does not move outside of this region. In our assay, this condition is fulfilled since the protein of interest is photoactivated in a small spot (diameter 1 μm) within a significantly larger zone containing the damage (6 μm^2) (Tomas et al., 2012). These caveats concerning the temporal and spatial equilibrium criteria apply in general to all methods combining microirradiation with fluorescence photoperturbation.

An aspect that needs to be specifically considered when attempting to model data from non-linear photoactivation experiments concerns the shape of the photoactivated volume. Due to the three-dimensional confinement of non-linear excitation the signal intensity is not uniform along the optical axis. Thus, the conditions for a two-dimensional approximation are not fulfilled strictly (point 2). On the other hand, the small spot size minimizes the influence of nuclear geometry which may therefore be neglected without inducing significant errors (point 3).

An overview of recently proposed approaches for the analysis of FRAP and photoactivation experiments specifying their most important features is given in **Table 4**. The study of Mazza et al. addresses the point of the three-dimensionally confined non-linear excitation volume and proposes an analytical solution of the diffusion equation for a two-photon photoactivated/bleached circular area of variable size. The method enables to determine diffusion coefficients over a wide range of values, but binding,

i.e., a reaction dominated protein dynamics, is only taken into account as an immobile fraction. A solution for the reaction-diffusion model is not given (Mazza et al., 2008). Sprague et al. develop an analytical solution of the reaction-diffusion model which applies to a spatially localized cluster of binding sites and use it to simulate binding of the glucocorticoid receptor to an array of promoter sites in live cells. This approach has the advantage of taking into account a non-homogenous distribution of binding sites as it occurs in microirradiated cell nuclei. However, it is only applicable if the size of the cluster (the damaged area) is smaller than the bleached/photoactivated region. This model has been recently applied to describe the behavior of Nbs1 and Mdc1 at sites of DNA damage introduced by charged particle irradiation (Sprague et al., 2006; Tobias et al., 2013).

Numerical solutions of the reaction-diffusion model provide a more accurate approach to describe protein dynamics. In their study, Beaudouin et al. present a method that includes both the real geometry of the nucleus and an inhomogeneous distribution of binding sites and is independent of the shape of the bleached/photoactivated region. The differential equations are solved numerically using a finite difference method. The approach was validated for the photoactivation of five different chromatin proteins (Beaudouin et al., 2006). It is interesting to note that in the case of histone H1, these authors do not detect

Table 4 | Selection of recently proposed analysis models for FRAP and photoactivation (PA) experiments.

Reference	Model	Solution/dimension	Bleached/photoactivated area	Remarks
Sprague et al., 2004	Reaction-diffusion	Analytical; 2D	Circular	Laplace transform of the solution; simplified solutions in explicit form for different values of association and dissociation rates
Carrero et al., 2004	Reaction-diffusion, and compartment model	Analytical; 1D/2D	Line/rectangular	Explicit solution for a bleached stripe; considers biphasic behavior of diffusion and binding
Phair et al., 2004	Reaction dominant, no diffusion	Analytical; 2D	50% of the nucleus	Considers only binding, diffusion is neglected
Sprague et al., 2006	Reaction-diffusion	Analytical; 2D	Circular	Subnuclear compartments with different binding rates in axial direction can be regarded
Mazza et al., 2008	Pure diffusion and immobile fraction	Analytical; 2D/3D	Circular	Appropriate for multiphoton FRAP/PA
Kang and Kenworthy, 2008	Reaction-diffusion	Analytical; 2D	Uniform circle or Gaussian	Explicit solution (no Laplace transform as in Sprague et al.)
Beaudouin et al., 2006	Reaction-diffusion	Numerical; 2D	50% of the nucleus	Considers nuclear geometry and inhomogeneous distribution of binding sites
Calvert et al., 2007	Diffusion	Numerical; 3D	Gaussian ellipsoid	Multiphoton excitation, spherical boundary condition, model calculates intensity along a line
Stasevich et al., 2010	Reaction-diffusion	Numerical; 2D	Circular	Model calculates intensity along a line across the bleach profile

subpopulations with higher and lower mobility, contrarily to what was reported previously in FRAP studies (Raghuram et al., 2010; Stasevich et al., 2010). Since the bleaching laser may induce phototoxic effects including DNA damage (Dobrucki et al., 2007; Solarczyk et al., 2012) and the mobility of the protein is increased in the presence of such lesions (Tomas et al., 2012), the partial fluorescence recovery observed in FRAP experiments can be due to a change of the protein's mobility within the bleached area leading to a local decrease in concentration rather than to the presence of

an immobile fraction. These effects have to be considered when choosing experimental conditions for photoperturbation studies.

None of the currently available models addresses the issue of the temporal non-equilibrium of the system that is characteristic for mobility measurements performed subsequently to DNA microdamage, either via FRAP or photoactivation. Addressing this issue is a promising avenue for future work and a prerequisite for the proper quantitative description of the response of nuclear proteins to DNA damage.

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