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Review

Regulation of DNA Damage Responses by Ubiquitin and SUMO

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Ubiquitylation and sumoylation, the covalent attachment of the polypeptides ubiquitin and SUMO, respectively, to target proteins, are pervasive mechanisms for controlling cellular functions. Here, we summarize the key steps and enzymes involved in ubiquitin and SUMO conjugation and provide an overview of how they are crucial for maintaining genome stability. Specifically, we review research that has revealed how ubiquitylation and sumoylation regulate and coordinate various pathways of DNA damage recognition, signaling, and repair at the biochemical, cellular, and whole-organism levels. In addition to providing key insights into the control and importance of DNA repair and associated processes, such work has established paradigms for regulatory control that are likely to extend to other cellular processes and that may provide opportunities for better understanding and treatment of human disease.

Principles of Ubiquitylation and Sumoylation

Although initially discovered as a mechanism targeting proteins for destruction by the proteasome, ubiquitylation—the covalent attachment of the 76 amino acid residue protein ubiquitin to other proteins—is now also known to regulate protein activity, localization, and interactions (Bergink and Jentsch, 2009; Komander and Rape, 2012). In addition to ubiquitin, there are several ubiquitin-like proteins (UBLs) that are structurally related to ubiquitin. Ubiquitin and most UBLs are attached via their C-terminal glycine residues to target proteins by enzymatic reactions mediated by E1, E2, and E3 ligases (Figure 1). The most widely characterized UBL is the ~100 residue protein SUMO (small ubiquitin-related modifier). Eukaryotes usually contain a single type of ubiquitin that is encoded by multiple genes. By contrast, vertebrate cells possess two types of SUMO: SUMO-1 and the highly related proteins SUMO-2 and SUMO-3 (SUMO2/3) that appear to be functionally redundant. Simpler organisms such as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, however, contain a single SUMO (Smt3 and Pmt3, respectively). In mammals, ubiquitylation involves two E1s, over 35 E2s, and over 600 E3s, while sumoylation is mediated by a single heterodimeric E1, one E2 (UBC9/UBE2I), and approximately ten E3s.

Ubiquitin and SUMO are usually attached to substrates via isopeptide linkages between their C termini and the εNH_2 group of Lys residues on target proteins. In some cases, the target protein has a single ubiquitin or SUMO attached, while in others, several can be individually attached to multiple Lys residues on the target. Furthermore, because ubiquitin and some SUMOs possess modifiable lysine residues, conjugation cycles can often be repeated to produce polymeric chains (Bergink and Jentsch, 2009). In the case of ubiquitin, seven Lys residues can be used (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, and Lys63) along with the amino group of the N-terminal Met. SUMO2/3 but not SUMO1 bear internal sumoylatable Lys residues that can be used to form chains, while SUMO1 can be conjugated as a chain-terminator. Consistent with different ubiquitin and SUMO chains having different structures and physical properties, they have distinct functions. For example, while Lys48-, Lys29-, and Lys11-linked ubiquitin chains promote target-protein degradation by the proteasome, Lys63 chains generally regulate protein-protein interactions. There is also evidence for ubiquitin chains with mixtures of linkages (Komander and Rape, 2012), as well as chains containing both ubiquitin and SUMO (Praefcke et al., 2012).

Like other posttranslational modifications, sumoylation and ubiquitylation are reversible. While SUMO-protein isopeptide bonds are cleaved by a small family of peptidases (SENP1–SENP3 and SENP5–SENP7), there are ~100 deubiquitylating enzymes (also known as deubiquitylases or DUBs). DUBs are grouped into five families: ubiquitin C-terminal hydrolases (UCHs), ubiquitin-specific proteases (USPs) and ovarian tumor proteases (OTUs), the Josephins, and the Jab1/MPN/Mov34 family (JAMM/MPN+). The first four families are Cys proteases, whereas the latter comprises Zn^{2+}-dependent metalloproteases (Nijman et al., 2005). In addition to opposing ubiquitin/SUMO ligase activities, certain DUBs and SENPs process ubiquitin and SUMO precursors, and some DUBs are intrinsic components of the proteasome.

DNA Repair and the DNA Damage Response

Genome integrity is continuously undermined by exogenous and endogenously generated DNA-damaging chemicals, ionizing radiation (IR) and ultraviolet (UV) radiation, and by errors in DNA replication. To mitigate this, cells possess highly effective mechanisms—collectively called the DNA damage response (DDR)—to detect, signal, and repair DNA lesions. These processes have profound impacts on normal cell and organism physiology, with their deregulation or loss causing genome instability syndromes that are associated with cancer, stem

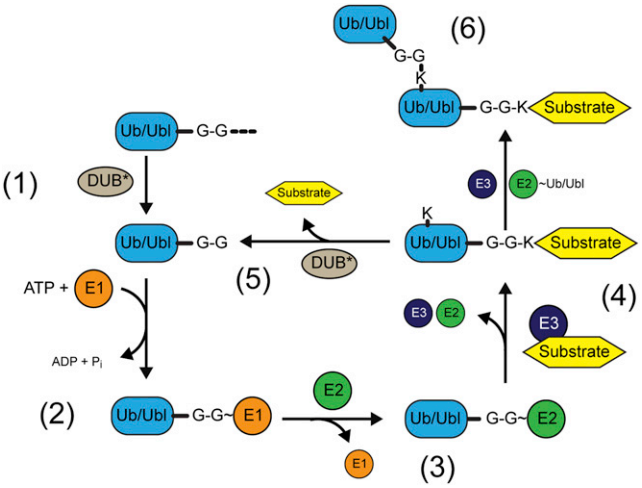

Figure 1. The Ubiquitin and UBL Conjugation Cycle
Ubiquitin and SUMO are produced as precursor polypeptides that are first processed to reveal a C-terminal diglycine motif (1). An E1 enzyme then uses ATP to convert this motif into a high-energy-bond containing adenylated derivative, which is short-lived, rapidly reacting with a Cys in the E1 to form a E1∼Ub or E1∼SUMO thioester intermediate (2). The ubiquitin or SUMO moieties are then coupled, via a transesterification reaction, onto a Cys residue in the E2 catalytic site to form E2∼Ub/SUMO intermediates (3). In most cases, an E3 ligase serves as a substrate adaptor, linking the charged E2 and the substrate (4). Ubiquitylation or sumoylation can be reversed by a DUB (for ubiquitin) or a SENP (for SUMO) (5) or the modification cycle can be repeated to produce chains of various topologies (6). The (*) indicates that other types of enzymes can remove UBL modifications.

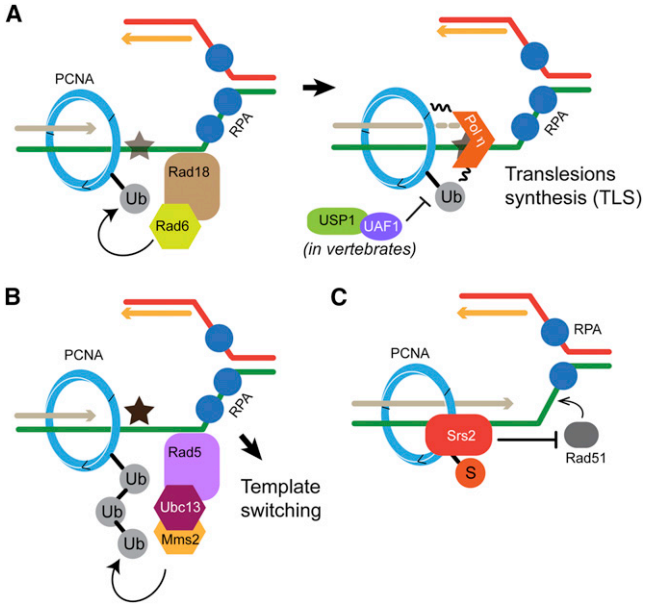

Figure 2. The roles of Ubiquitin and SUMO in Postreplication Repair
(A) PCNA monoubiquitylation on Lys164 occurs when replication forks encounter lesions (star). The single-stranded DNA is recognized by the Rad18 E3 ligase, which interacts with Rad6, an E2, to ubiquitylate PCNA. PCNA ubiquitylation recruits Y family polymerases such as Polη to bypass the lesion in the process termed translesion synthesis (TLS). In vertebrates the USP1-UAF1 DUB opposes PCNA monoubiquitylation.
(B) A second DNA damage tolerance pathway termed template switching occurs when PCNA is polyubiquitylated by the E3 Rad5 and the Ubc13/Mms2 dimeric E2. Template switching employs homologous recombination.
(C) In yeast, PCNA sumoylation on its Lys164 residue recruits Srs2, which acts as an antirecombinase by disrupting Rad51 nucleoprotein filaments.

cell exhaustion, developmental defects, infertility, immune deficiency, neurodegenerative disease, and premature aging (Jackson and Bartek, 2009).

Different DNA lesions are repaired by distinct systems. Thus, DNA double-strand breaks (DSBs) are repaired by nonhomologous end joining (NHEJ), alternative NHEJ, or homologous recombination (HR), UV-induced DNA lesions, and other bulky DNA adducts are repaired by nucleotide excision repair (NER), simpler base lesions are repaired by base-excision repair (BER) whose components and reactions overlap with those of single-strand break repair, and DNA base mismatches are corrected by mismatch repair (MMR), while the Fanconi anemia (FA) pathway repairs DNA crosslinks. Lesions are first recognized by proteins that trigger and coordinate the recruitment and activities of additional DNA repair components. DNA damage induction elicits cascades of posttranslational modifications, including phosphorylation, ubiquitylation, and sumoylation that orchestrate the aforementioned processes as well as additional aspects of the DDR, such as regulating deoxyribonucleotide supply and triggering of cell-cycle delays (cell-cycle checkpoints). These events are largely initiated by the apical DDR kinases ATM and ATR, whose importance in coordinating the DDR is illustrated by their mutation in Ataxia-telangiectasia (A-T) and Seckel syndrome, respectively (Durocher and Jackson, 2001; Kerzendorfer and O'Driscoll, 2009). In this review, we focus on the rapidly emerging functions of ubiquitin and SUMO in controlling various aspects of the DDR, with a particular emphasis on responses to DSBs, which are the most cytotoxic of all DNA lesions.

Ubiquitylation and Postreplication Repair

The first association between DNA repair and ubiquitylation arose when yeast Rad6, which functions in postreplication repair (PRR), was shown to be a ubiquitin E2 (Jentsch et al., 1987). PRR is a DNA-damage-tolerance pathway that allows replication past bulky DNA lesions and is orchestrated by ubiquitylation and sumoylation of the DNA polymerase processivity factor PCNA (Figure 2). Two subpathways comprise eukaryotic PRR: translesion synthesis (TLS), and a template-switch mechanism associated with HR (Ulrich, 2011). An early step in yeast PRR is PCNA monoubiquitylation by the RING-type E3, Rad18, in conjunction with the E2, Rad6 (Hoege et al., 2002; Stelter and Ulrich, 2003). PCNA is primarily monoubiquitylated on Lys164, with this modification being recognized by specialized TLS polymerases such as Polη, Pol ι, Pol κ, and Polζ via their ubiquitin-binding domains (UBDs) of the UBM and UBZ families in conjunction with motifs such as PIP boxes that recognize other features of PCNA (Lehmann, 2011). Unlike canonical high-fidelity polymerases, the catalytic sites of TLS polymerases can synthesize over and past DNA lesions but usually at the cost of fidelity, thus making them error prone. Yeast PCNA can also be polyubiquitylated on Lys164 by Rad5 (Hoege et al., 2002), an E3 ligase that cooperates with a dimeric E2 Ubc13-Mms2. Rad5-Ubc13-Mms2 yields Lys63-linked ubiquitin chains on PCNA that promote the template-switching pathway that involves the newly synthesized

sister chromatid and the HR machinery. While it is still unclear how PCNA ubiquitylation promotes template switching, the mammalian ZRANB3 translocase was recently identified as an effector of PCNA polyubiquitylation (Zeman and Cimprich, 2012).

While differences in PRR may exist between yeast and man, the pathway has been generally evolutionarily conserved, with mammals having counterparts of Rad6 (human HR6A/UBE2A and HR6B/UBE2B), Rad18 (RAD18), and Rad5 (SHPRH and HLTF). Knockout or depletion of *RAD18* in a variety of species results in defective PRR, PCNA monoubiquitylation, and accumulation of TLS polymerases such as Polη at sites of replication fork blockage (Lee and Myung, 2008). Furthermore, small interfering RNA depletion studies suggest that human HLTF and SHPRH contribute to PCNA polyubiquitylation in response to fork-causing lesions (Motegi et al., 2008) and in the suppression of mutagenesis (Lin et al., 2011), phenotypes consistent with functions for these E3 ligases in error-free PRR.

In addition to being ubiquitylated, budding yeast PCNA is sumoylated on Lys164 (and to a lesser degree on Lys127) by the E3 Siz1 and the E2 Ubc9 (Pfander et al., 2005; Stelter and Ulrich, 2003). PCNA sumoylation prevents unscheduled recombination during DNA replication by recruiting Srs2, a UvrD-type helicase that can strip the key HR protein Rad51 from chromatin (Krejci et al., 2003; Pfander et al., 2005; Veaute et al., 2003). Srs2 harbors PCNA-binding PIP and SUMO-interaction motif (SIM) regions that simultaneously engage sumoylated PCNA (Armstrong et al., 2012). While PCNA sumoylation is difficult to detect in human cells, an Srs2-like protein, PARI, has recently been described (Moldovan et al., 2012).

Control of NER by Ubiquitylation

NER repairs bulky DNA base adducts and ultraviolet light-induced lesions. Inherited defects in NER factors yield pathologies that include xeroderma pigmentosum (XP), Cockayne syndrome (CS), and trichothiodystrophy (TTD), which are characterized by sun hypersensitivity, skin cancer predisposition (in the case of XP), cognitive impairments, premature aging, or developmental defects (Hoeijmakers, 2009). NER comprises two main pathways: global genome repair (GG-NER) that operates on all nuclear DNA, and transcription-coupled repair (TC-NER) that specifically targets the template strand of transcribed genes. During human GG-NER, DNA lesions can be detected independently by two complexes, DDB1-DDB2/XPE and XPC-RAD23 (Scrima et al., 2011) (Figure 3). However, DDB1-DDB2 plays a unique role in NER owing to the observation that DDB1-DDB2 is required for the effective recruitment of XPC to chromatin (Fitch et al., 2003). Mechanistically, DDB2-DDB1 forms an E3 ligase in association with CUL4A/B that mediates monoubiquitylation of histones and polyubiquitylation of DDB2 and XPC (Scrima et al., 2011). While autoubiquitylated DDB2 is targeted for degradation, XPC is not because it is protected from proteasome action by RAD23, a proteasome-interacting protein (El-Mahdy et al., 2006; Sugasawa, 2006; and references therein).

When RNA polymerase II (RNAP II) stalls upon encountering a DNA lesion, two independent cascades can be triggered. The first event is TC-NER, and the second is the ubiquitylation, extraction, and degradation of RNAP II from chromatin (Figure 3). TC-NER is dependent on CSB (ERCC6), a SWI/SNF family protein that associates with RNAP II (Gaillard and Aguilera, 2013). In addition to possessing chromatin-remodeling activity, CSB recruits CSA (ERCC8) to sites of DNA damage, the latter forming an E3 with DDB1 and CUL4. The action of CSB and CSA may be to license the TC-NER process, which includes RNAP II backtracking and subsequent recruitment of the core NER machinery (Gaillard and Aguilera, 2013). The identity of the key ubiquitylation event that initiates TC-NER is still unknown, but RNAP II and CSB ubiquitylation are possibilities. In that regard, CSB possess a functionally important UBD, suggesting that CSB recognizes this key ubiquitylation event (Anindya et al., 2010). Furthermore, the DUB USP7 is recruited to stalled polymerases by UVSSA, the product of a gene mutated in a CS-like UV-sensitivity syndrome (Cleaver, 2012). UVSSA-USP7 interacts with RNAP II and delays the CSA-dependent degradation of CSB by the proteasome.

The degradation of RNAP II by the proteasome can be seen as a last-resort measure and provides a unique case study for the role of ubiquitin chain editing. In yeast, these processes involve the Rsp5 E3 (NEDD4 in mammals), which catalyzes Lys63-linked ubiquitin chain formation on RNAP II (Anindya et al., 2007; Wilson et al., 2013). These chains are trimmed down by Ubp2, a DUB, resulting in monoubiquitylated RNAP II. Lys48-linked ubiquitin chains are then built from monoubiquitylated RNAP II by an Elongin/Cullin 3 complex, which can then promote RNAP II degradation after its extraction from chromatin with the Cdc48 segregase, the yeast VCP/p97 homolog (Harreman et al., 2009; Verma et al., 2011; Wilson et al., 2013).

Ubiquitin-Based DSB Signaling by RNF8 and RNF168

An important paradigm for ubiquitin and SUMO acting in intracellular signaling is provided by the orchestrated recruitment of proteins such 53BP1 and the tumor suppressor BRCA1 onto chromatin surrounding DSB sites (Lukas et al., 2011) (Figure 4). These events are initiated by phosphorylation of the histone variant H2AX (yielding γH2AX), which is recognized by MDC1 (Stucki et al., 2005). MDC1 is then phosphorylated by the DSB-responsive kinase ATM, with these phospho-sites being bound by the FHA domain of the RING-E3 ligase RNF8 (Huen et al., 2007; Kolas et al., 2007; Mailand et al., 2007). RNF8 then mediates ubiquitylation of proteins at DSB sites in a manner promoted via interactions with the large HECT-type ligase HERC2 (Bekker-Jensen et al., 2010). The RING-E3 ligase RNF168 is then recruited by its UBDs, recognizing RNF8 ubiquitylation products and products of its own activity. The UBDs of RNF168 are not equivalent and are integrated in functional modules containing targeting motifs, the LRMs that are also present on RAD18 and RAP80 (Panier et al., 2012). The primary outcome of RNF8/RNF168-dependent ubiquitylation is recruitment and/or retention of DSB repair and signaling factors on chromatin surrounding the DNA lesion, which include 53BP1, RAD18, BRCA1, the RAP80 complex (also known as BRCA1-A), HERC2, BMI1, RIF1, RNF169, NPM1, FAAP20, and NIPBL (Lukas et al., 2011). At the functional level, RNF8/RNF168-dependent ubiquitylation promotes NHEJ during immunoglobulin class switching and dysfunctional telomere fusion (Kracker and Durandy, 2011;

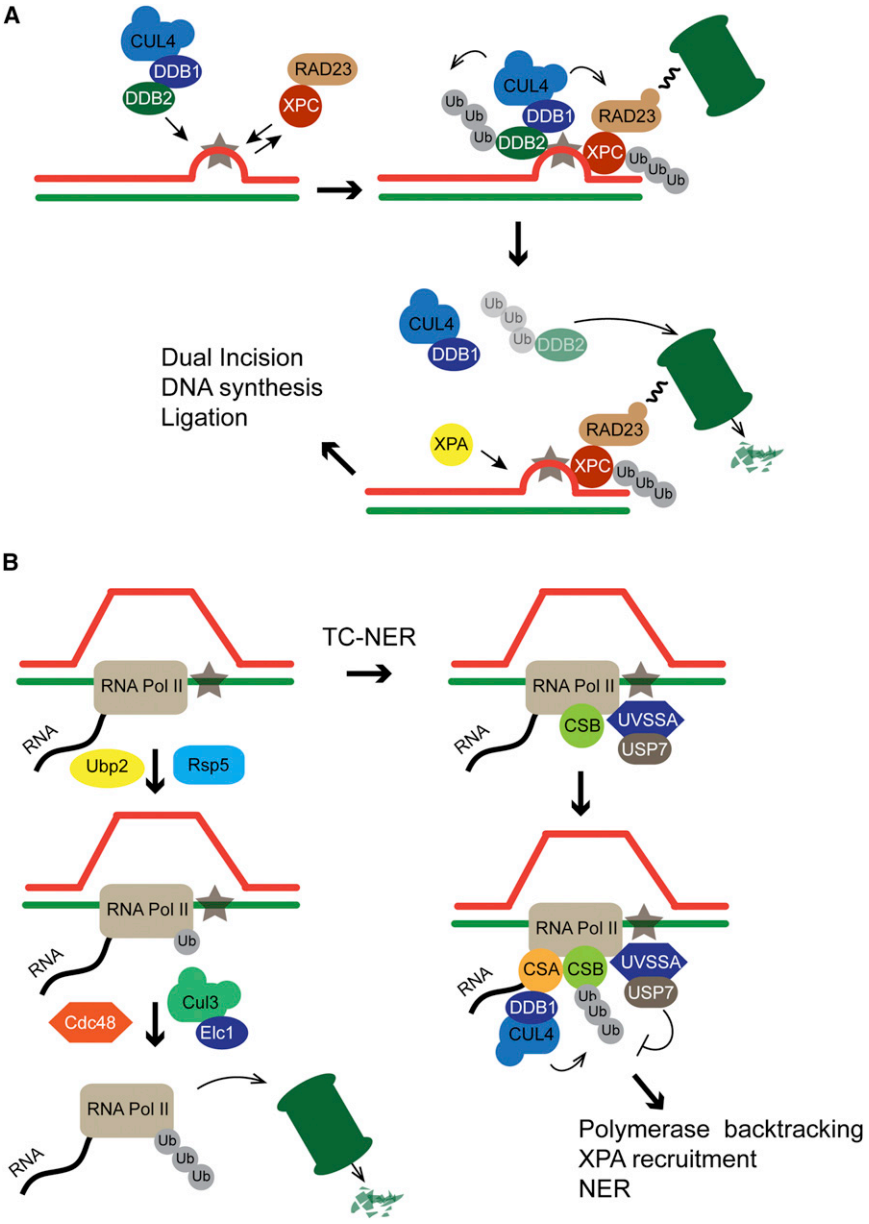

Figure 3. The Role of Ubiquitin in Nucleotide Excision Repair

(A) GG-NER promotes the repair of bulky lesions on genomic DNA. The lesions can be recognized by the DDB1-DDB2 and XPC-RAD23 complexes. DDB1-DDB2 forms an E3 with CUL4 and RBX1 (not shown), which leads to DDB2 autoubiquitylation, resulting in its degradation, and XPC polyubiquitylation. XPC is stabilized through its interaction with RAD23, which contains a ubiquitin-like domain that interacts with the proteasome. This allows the subsequent steps of NER, such as the recruitment of XPA.

(B) TC-NER repairs bulky lesions occurring on the transcribed DNA strand. RNA polymerase II acts as a lesion sensor. Two independent pathways can occur when RNA polymerase stalls after a lesion encounter. First (going right), TC-NER can be activated, with the CSB and CSA proteins playing a critical role in TC-NER. CSB associates with RNA polymerase and recruits CSA, which forms a CUL4-based E3 with DDB1. The exact nature of the critical substrate of the E3 associated with CSA is not known, but CSB is ubiquitylated and its degradation is delayed by USP7, which is brought to the stalled polymerase by UVSSA. Second (going down), the RNA polymerase can be ubiquitylated, extracted from chromatin, and degraded by the proteasome as a last resort. This pathway, in yeast, is initiated by Rad26-Def1 (not shown), which leads to the Rsp5-dependent ubiquitylation of RNA polymerase. Rsp5 can promote Lys63-ubiquitin chains but the DUB Ubp2 trims these down. Monoubiquitylated RNA polymerase is then a substrate of an Elongin-Cullin 3 complex. The Lys48-linked ubiquitin chains on RNA polymerase enable Cdc48 to extract the stalled polymerase from chromatin, which then leads to its degradation.

Peuscher and Jacobs, 2011; Rai et al., 2011). In addition to NHEJ, the RNF8 pathway can also promote HR. These repair defects likely contribute to the clinical phenotypes observed in individuals with inactivating mutations in *RNF168*, which are afflicted with an immunodeficiency and cellular radiosensitivity syndrome, RIDDLE, that is related to A-T (Stewart et al., 2009).

Significantly, the RNF8 pathway is turned off during mitosis, perhaps because chromatin ubiquitylation is incompatible with mitotic progression (Giunta et al., 2010). In another striking example of regulation, it recently emerged that RNF168 is a limiting factor in the RNF8 pathway, with the E3 ubiquitin ligases TRIP12 and UBR5 collaborating to regulate RNF168 levels, thereby preventing excessive histone ubiquitylation at DSB sites (Gudjonsson et al., 2012). It will be interesting to determine how TRIP12 and UBR5 affect RNF168 levels and whether any physiological conditions affect this regulation. In this regard, TRIP12 contains a WWE domain, which in other proteins binds to poly(ADP) ribose, suggesting that RNF168 levels might be controlled by poly(ADP) ribosylation. Herpes simplex virus (HSV) infection also regulates the RNF8 pathway, with the HSV ICP0 protein—which is necessary for the transition between the viral latent and lytic phases—being an E3 that targets RNF8 and RNF168 for degradation (Chaurushiya et al., 2012; Lilley et al., 2010). Specifically, ICP0 targets RNF8 for degradation through a "reverse-degron" mechanism mediated by a phospho-dependent interaction between ICP0 and the RNF8 FHA domain (Chaurushiya et al., 2012). This RNF8 and RNF168 degradation not only obfuscates the DDR initiated by the linear HSV DNA but also relieves RNF8/168-dependent transcriptional repression of the viral genome.

While early work pointed to H2A-type histones being primary RNF8/168 targets, mutational studies suggested that RNF8/RNF168 target sites were distinct from the canonical H2AK119 ubiquitylation sites (Huen et al., 2007). Indeed, RNF168 was

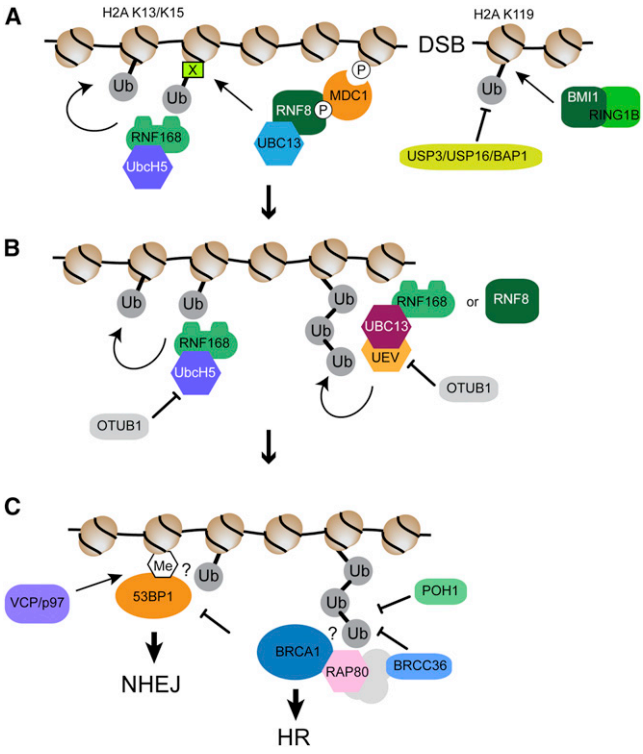

Figure 4. The Role of Ubiquitin in the Chromatin-Based Response to DNA Double-Strand Breaks

(A) DSBs trigger the ATM-dependent phosphorylation of H2AX, which is read by MDC1. ATM can also phosphorylate MDC1 to promote the recruitment of RNF8, an E3 ligase. RNF8 ubiquitylates an unknown factor on chromatin (X). This ubiquitylation is then read by the N-terminal ubiquitin binding domains of RNF168. RNF168 ubiquitylates H2A, which provides a second recruitment signal for RNF168. In addition to RNF8/168, DSBs stimulate the recruitment of the BMI1-RING1B, which ubiquitylates H2A on the C terminus. Also shown are DUBs that antagonize this ubiquitylation event.

(B) OTUB1 antagonizes RNF168-dependent ubiquitylation by binding to E2s. RNF8 and RNF168 also stimulate the formation of Lys63-linked ubiquitin chains on chromatin.

(C) The RNF168-dependent ubiquitylation of chromatin leads to the recruitment of multiple effectors that include 53BP1, which promotes NHEJ, and BRCA1, which promotes HR.

recently shown to target Lys13/15 within the histone H2A N terminus (Gatti et al., 2012; Mattiroli et al., 2012). It will be important to determine whether and how H2A Lys13/15 ubiquitylation may promote recruitment of proteins to chromatin flanking DSB sites and how this may functionally cooperate with other DSB-responsive histone modifications. In this regard, while H2A Lys13, and Lys15 are located far from H2AK119 on the nucleosome core particle, they are close to H2BK120, a residue ubiquitylated by the RNF20 and RNF40 E3 ligases in collaboration with the RAD6 E2 (Zhu et al., 2005). RNF20 and RNF40 are also recruited to DSB sites via ATM-dependent mechanisms, to induce H2B monoubiquitylation (Moyal et al., 2011; Nakamura et al., 2011). These observations may help explain why *Rnf8*-deficient mouse embryo fibroblasts display reduced steady-state H2B K120 ubiquitylation (Wu et al., 2009). Given the role of mammalian H2B ubiquitylation in regulating transcription and chromatin compaction (Weake and Workman, 2008), the potential crosstalk between H2A K13/K15 and H2B K120 ubiquitylation might at least in part explain RNF8/RNF168-dependent transcriptional silencing triggered near DSBs (Shanbhag et al., 2010).

Histone ubiquitylation stimulated by DNA lesions is not limited to H2A K13/K15 and H2B K120. Indeed, the E3 BMI1-RING1B accumulates at DSB sites, where it is proposed to locally increase H2A/H2AX K119 monoubiquitylation, which may participate in DNA-damage-induced transcriptional silencing (Gieni et al., 2011; Shanbhag et al., 2010). Furthermore, recent proteomic work found that all core histones along with histones H1, H2AZ, H2AX, and macro-H2A are ubiquitylated at multiple sites (Kim et al., 2011; Wagner et al., 2011). It will clearly be of interest to determine whether any of these modifications affect or are affected by responses to DNA lesions.

Regulating DSB Responses by 53BP1 and BRCA1

53BP1 and BRCA1 are the two main effectors of the RNF8 pathway, yet they have diametrically opposed functions: 53BP1 opposes DNA end resection, an activity that promotes DSB repair by NHEJ (Noon and Goodarzi, 2011), while BRCA1 promotes HR and is somehow linked to initiation of end resection (Li and Greenberg, 2012). BRCA1 and 53BP1 are thus essentially engaged in a tug of war that determines commitment to NHEJ or HR. This functional antagonism has profound implications for our understanding of BRCA1 function as a tumor suppressor, since inactivation of 53BP1 suppresses the lethality, tumorigenesis, and sensitivity to most genotoxins associated with BRCA1 loss of function (Bouwman et al., 2010; Bunting et al., 2010). A provocative implication of this work is that the function of BRCA1 might act as a competitor or inhibitor of 53BP1, particularly in S/G2 phases, when HR is upregulated (Chapman et al., 2012).

The mechanism for 53BP1 recruitment to DSB sites has been puzzling because it does not possess recognizable ubiquitin-binding regions. Instead, it contains a tandem Tudor domain that binds mono- or dimethylated histone H4 Lys20 (H4K20me1/2) and a tandem BRCT domain involved in protein-protein interactions (Botuyan et al., 2006). Notably, the Tudor but not the BRCT region of 53BP1 is needed for its accumulation at DSB sites (Pryde et al., 2005). Recent work showed that the proteins L3MBTL1 and JMJD2A bind to H4K20me1/2 but are removed from chromatin upon DNA damage induction (Acs et al., 2011; Mallette et al., 2012). L3MBTL1 removal requires the segregase VCP/p97, which is targeted to DSB sites through K48-linked ubiquitin and its cofactor NPL4 (Acs et al., 2011), whereas the RNF8 pathway promotes JMJD2A degradation (Mallette et al., 2012). A role of VCP/p97 in 53BP1 retention at DSB sites was also identified by Meerang et al. (2011), although the conclusions reached regarding mechanism differed. While a model for ubiquitylation simply acting to remove JMJD2A/L3MBTL1 is attractive, it is difficult to reconcile it with the constitutive, Tudor-dependent association of 53BP1 with chromatin (Bothmer et al., 2011) and with H2A K13/K15 ubiquitylation being critical for 53BP1 DSB recruitment (Mattiroli et al., 2012). The mechanism(s) for 53BP1 recruitment at DSB sites will likely remain unresolved until the biochemical reconstitution of its RNF168-dependent chromatin binding is achieved.

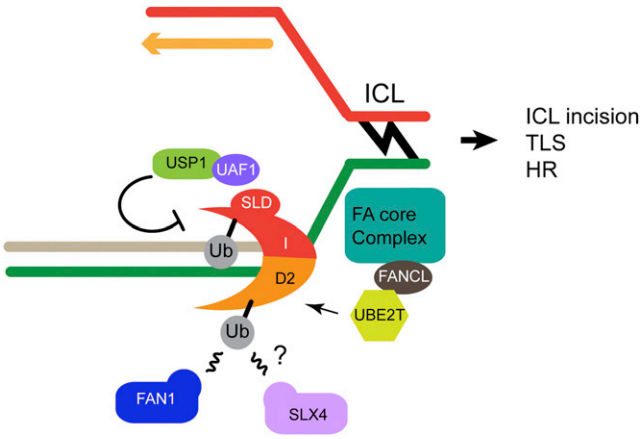

Figure 5. The Role of Ubiquitin in the FA Pathway
ICLs are potent fork-blocking lesions. DNA replication fork stalling, along with FANCM and associated proteins (not shown), stimulates the recruitment of the multi-subunit FA core complex, which is an E3 ligase. The catalytic subunit of this E3 is FANCL and its E2 is UBE2T. The FA core complex monoubiquitylates FANCD2-FANCI. FANCD2-FANCI ubiquitylation is read by FAN1 and perhaps SLX4, which both contain a UBZ4-type ubiquitin binding domain. The USP1-UAF1 DUB reverses FANCD2-FANCI ubiquitylation and is essential for the FA pathway. The recruitment of nucleases enables incision, unhooking of the lesion, TLS, and HR.

BRCA1 forms a dimeric RING-type E3 with BARD1 and, in addition to its role in HR, has also been linked to transcription-coupled DNA repair, DNA crosslink repair, and checkpoint control. While BRCA1 recruitment at DSB sites clearly relies on RNF8- and RNF168-dependent formation of ubiquitin conjugates (Lukas et al., 2011), exactly how BRCA1 recognizes such ubiquitin conjugates is still under intense investigation. Long-term maintenance of BRCA1 at DSB sites depends on its interaction with the RAP80 complex—and the RAP80 ubiquitin-binding UIM modules—but RAP80 is dispensable for the initial accumulation of BRCA1 at DSB sites (Hu et al., 2011; Yin et al., 2012b). There is also vigorous debate about whether BRCA1 E3 ligase activity is crucial for DSB repair and tumor suppression, with the balance of opinion tilting toward it not being essential for DSB repair by HR (Li and Greenberg, 2012; Shakya et al., 2011; Zhu et al., 2011). Indeed, a mutation in the RING domain (*Brca1*^{I26A}) that only affects its interaction with E2 conjugating enzymes results in cells that are proficient in repairing a targeted DSB by HR, along with mice that are phenotypically normal with wild-type tumor latency (Li and Greenberg, 2012; Shakya et al., 2011). However, a similar mutation in chicken DT40 cells caused hypersensitivity to DNA damage caused by the topoisomerase I poison camptothecin (Sato et al., 2012). Additional work is clearly needed to define the specific function(s) for BRCA1 E3 ligase activity.

Ubiquitin Control of the Fanconi Anemia Pathway

Fanconi anemia (FA) is a rare recessive genetic disorder characterized by developmental abnormalities, cancer predisposition, progressive bone-marrow failure, and defective repair of DNA interstrand crosslinks (ICLs) that prevent transcription and DNA replication (Garner and Smogorzewska, 2011; Kim and D'Andrea, 2012). There are currently 15 known genes whose biallelic mutations yield FA (FANCA to FANCP), with their products functioning in the three prime events of the FA pathway that occur in S phase: ICL recognition and excision, translesion synthesis, and HR-mediated repair (Knipscheer et al., 2009). The FA pathway is activated when replication forks encountering ICLs trigger chromatin association of the FA core complex (Figure 5). The FANCL subunit of this complex is a RING-type E3 ligase that functions with the E2, UBE2T, to monoubiquitylate both subunits of the heterodimeric FANCD2-FANCI complex, which then provides a platform for coordinating repair processes (Joo et al., 2011). Specifically, in addition to ubiquitylation potentially helping anchor FANCD2-FANCI on chromatin, ubiquitylated FANCD2 is recognized by the UBZ4 UBD present in the structure-specific nuclease FAN1, although an interaction with the nuclease scaffold SLX4 (FANCP) has also been proposed (Sengerová et al., 2011). This promotes recruitment of these factors to sites of DNA damage, with the ensuing nucleolytic incisions triggering further stages of repair that include TLS and HR events discussed elsewhere in this review. Additional connections between the FA pathway and ubiquitin are highlighted by work demonstrating that the FA core-complex-associated factor, FAAP20, contains a UBD that binds RNF8-mediated ubiquitylations and cooperates with RNF8 to promote recruitment of the FA core complex and FANCD2 to ICLs (Yan et al., 2012).

Deubiquitylases Acting in the DDR

A well-characterized DUB is USP1, the prime FANCD2 deubiquitylase, whose inactivation recapitulates many FA phenotypes, implying that both ubiquitylation and deubiquitylation must be appropriately controlled for the FA pathway to operate effectively (Kim and D'Andrea, 2012). In this regard, the USP1-activating factor, UAF1/WDR48, possesses two tandem SUMO-like domains that mediate interactions with a SIM-related sequence in FANCI, thus targeting USP1 to FANCD2/FANCI (Yang et al., 2011) (Figure 5). The DUBs USP3, USP16, BRCC36, POH1, and OTUB1 are associated with negative regulation of the RNF8 pathway, with USP3 and USP16 being first linked to this pathway through their ability to oppose H2A ubiquitylation (Weake and Workman, 2008) and by USP3 overexpression blocking RNF168 accumulation at DSB sites (Doil et al., 2009). USP16, on the other hand, opposes RNF8/RNF168-mediated, DSB-induced transcriptional silencing (Shanbhag et al., 2010). BRCC36 (BRCC3)—a JAMM/MPN(+) isopeptidase that displays strong selectivity for K63-linked ubiquitin chains and that is a component of the BRCA1-RAP80 complex—accumulates at DSB sites downstream of RNF8/RNF168 (Cooper et al., 2009; Dong et al., 2003; Shao et al., 2009; Sobhian et al., 2007; Wang and Elledge, 2007). In addition, a second JAMM/MPN(+) protein with specificity against K63-linked ubiquitin chains, the proteasome-associated POH1/PSMD14 DUB, has been linked to negative regulation of the RNF8 pathway (Butler et al., 2012). Collectively, these findings suggest that proteasome-associated POH1 and BRCA1/RAP80-associated BRCC36 may collaborate to restrict K63-linked, RNF8/RNF168-dependent polyubiquitylation at DSB sites. Notably, the OTU family DUB, OTUB1, is a negative

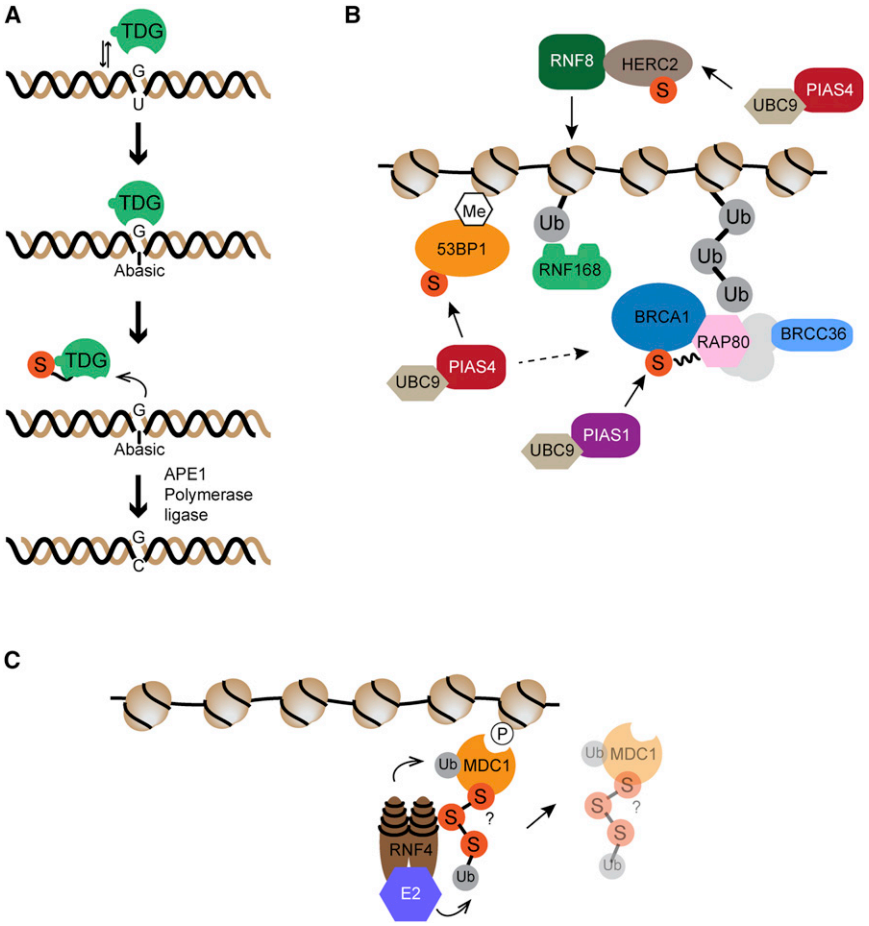

Figure 6. Examples of the Role of SUMO in Multiple DNA Damage Response Pathways

(A) Thymine-DNA glycosylase (TDG), which acts during BER, is inhibited by the product of its reaction. TDG sumoylation produces a conformational change that enables it to leave DNA.

(B) Sumoylation acts at multiple steps during the chromatin-based DSB response. See the main text for details.

(C) Sumoylation can also stimulate ubiquitylation through the action of STUbLs, E3 ligases that are recruited to substrates by recognizing sumoylation. In the shown example, RNF4 promotes the turnover of MDC1 from chromatin during the DSB response.

regulator of RNF168-dependent ubiquitylation, but, surprisingly, this is independent of the OTUB1 isopeptidase activity (Nakada et al., 2010). Instead, OTUB1 binds and inhibits the RNF168-associated E2s (UBC13/UBE2N and E2s of the UBE2D/UBE2E families) through assembly of a pseudocleavage product in its catalytic site (Juang et al., 2012; Wiener et al., 2012). DUBs undoubtedly affect many other DDR processes, as highlighted by USP1-UAF1 also regulating PCNA ubiquitylation and TLS (Huang et al., 2006). Other notable DUBs are USP47, which affects BER by modulating DNA polymerase β levels (Parsons et al., 2011), the tumor suppressor BAP1, which regulates H2A K119ub (Scheuermann et al., 2010), and USP7, which regulates the stability of multiple DDR proteins, including p53 and ERCC6 (Fraile et al., 2012). Given their likely tractability to small-molecule drug development, it will be interesting to assess the potential of various DUBs as targets for pharmacological intervention.

Ubiquitylation Controls p53 in Response to DNA Damage

A prime executer of DNA damage signaling in vertebrates is the transcription factor p53, mutations in which are associated with various human cancers. Extensive work by many laboratories has established that p53 levels and function are regulated in response to DNA damage, with ubiquitylation and sumoylation playing crucial roles in these events. Due to the breadth of such research, we are only able to review it here in a cursory manner (for a more comprehensive review, see Lane and Levine, 2010). In brief, over ten ubiquitin E3s have been linked to p53 regulation, with most research having been focused on the RING E3, Mdm2/HDM2 (in mouse and human, respectively) (Brooks and Gu, 2011). Mdm2 functions as a homodimer, or as a heterodimer with the related E3 Mdmx/HDMX, to bind to and ubiquitylate p53, thus promoting proteasome-mediated p53 degradation and helping keep p53 levels low under normal conditions. In response to DNA damage, however, various modifications on p53, Mdm2, and their regulators, including those catalyzed by and promoted by the checkpoint kinases ATM, ATR, CHK1, and CHK2, inhibit p53 ubiquitylation and degradation and promote p53 transcriptional activity, allowing it and its cofactors to induce target genes. These genes in turn trigger various responses, including cell-cycle delays, induction of DNA repair proteins, and, in some cases permanent cell-cycle arrest or apoptosis. Furthermore, recent work has highlighted how various DUBs, including USP7/HAUSP, USP10, USP29, and USP42, play important roles in modulating p53 levels and activity (Hock et al., 2011).

A Paradigm for Sumoylation in BER

Sumoylation was first firmly linked to DNA repair by studies on BER (Figure 6), a pathway that is initiated by various base lesions being recognized by DNA glycosidases, proteins that enzymatically remove the lesions, yielding abasic sites that are then processed and repaired. Thymine-DNA glycosylase (TDG) removes thymine or uracil from G:T or G:U mismatches that arise through deamination of 5-methylcytosine or cytosine, respectively. Biochemical studies established that, like certain other DNA glycosylases, TDG strongly binds to the abasic site it generates, with such product inhibition impairing further steps in BER. Notably, SUMO1 conjugation in the TGD C-terminal region triggers interactions between this and SIMs elsewhere within

the protein, leading to a conformational change in the TDG N terminus that reduces product binding, thereby enhancing its enzymatic turnover (Baba et al., 2005; Steinacher and Schär, 2005). Such studies suggested a molecular handover model wherein unconjugated TDG binds and mediates base hydrolysis, with subsequent SUMO binding and sumoylation of TDG promoting its dissociation. This promotes the handover of the abasic BER intermediate to the APE1 endonuclease that mediates the next step of the repair process.

SUMO and Ubiquitin Crosstalk in DSB Repair

A well-characterized, direct link between sumoylation and DSB responses came through studies on *S. cerevisiae* Rad52, a key HR factor. Rad52 is sumoylated in a manner requiring the Mre11 complex, Ubc9, and the E3 Siz2 that is related to mammalian PIAS proteins (see below). In addition to this sumoylation protecting Rad52 from degradation, it promotes Rad52 exclusion from nucleoli, thus preventing inappropriate recombination between repetitive ribosomal DNA sequences (Torres-Rosell et al., 2007). Sumoylation also controls yeast HR in other ways. Cohesin is a multiprotein complex that encircles sister chromatids, thereby promoting equal chromosome distribution during mitosis and postreplicative sister-chromatid recombination. In *S. cerevisiae*, in addition to cohesion generation during S phase, it is further enforced locally and globally upon DSB induction in G2/M. The cohesin subunit Mcd1 is sumoylated upon DSB formation in a manner that promotes DNA-damage-induced cohesion and that largely relies on the SUMO E3 Mms21 (Nse2), which is part of the cohesin-related Smc5-Smc6 complex (McAleenan et al., 2012). Related mechanisms might operate in vertebrates because human Smc5-Smc6 is recruited to DNA damage sites, where it promotes sister chromatid HR, at least in part by MMS21/NSE2's sumoylation of the human Mcd1 ortholog SCC1 and counteracting the negative cohesin regulator WAPL (Wu et al., 2012). The demonstration of DNA-damage-induced sumoylation of various yeast HR, NHEJ, BER, NER, MMR, and checkpoint components highlights how sumoylation is likely to impact various components of many DDR processes (Cremona et al., 2012). Indeed, recent work in *S. cerevisiae* by (Psakhye and Jentsch, 2012) has established that while sumoylation of various HR factors is collectively crucial for effective DSB repair, loss of any one individual sumoylation has little effect. It seems likely that this principle of "protein group modification" will also apply to sumoylation in additional DNA repair pathways.

Links between sumoylation and DSB responses in mammalian cells came through the findings that SUMO1, SUMO2/3, UBC9, and the PIAS and MMS21 SUMO E3s accumulate at sites of DSBs or replication stalling (Galanty et al., 2009; Morris et al., 2009) (Figure 6). Moreover, PIAS4 inactivation markedly impaired RNF168 accrual and K63-ubiquitin accumulation together with 53BP1 and BRCA1 recruitment at DSB sites, while PIAS1 depletion only prevented RAP80 and BRCA1 accumulation. Accordingly, PIAS1 or PIAS4 depletion markedly impaired DSB repair by HR and NHEJ and caused hypersensitivity toward DSB-generating agents (Galanty et al., 2009; Morris et al., 2009). Significantly, while RNF8 or RNF168 depletion did not prevent PIAS1/4 recruitment to DNA damage sites, it markedly reduced SUMO1 and SUMO2/3 accrual. This is probably because RNF8/168 mediate the recruitment of factors such as BRCA1 and 53BP1 that are then sumoylated, although direct effects of RNF8/RNF168 on PIAS1/PIAS4 activities are also possible. Furthermore, although PIAS1/4 are not required for RNF8 recruitment to DNA damage sites, PIAS4 but not PIAS1 depletion impaired RNF168 recruitment. Notably, RNF168 and HERC2 are sumoylated in a DNA-damage- and PIAS4-dependent manner, with PIAS4 depletion reducing RNF168 levels, impairing HERC2 binding to RNF8, and abrogating IR-induced HERC2 accrual on chromatin (Danielsen et al., 2012). BRCA1 is sumoylated by PIAS1 and/or PIAS4 once localized at DSB sites, which enhances BRCA1 ubiquitin ligase activity (Morris et al., 2009), possibly via sumoylation helping BRCA1 to productively associate with E2 enzymes and/or SIM-containing target proteins. Additional functions of PIAS1 and PIAS4 are likely mediated by PIAS1/4-dependent sumoylation of factors such as 53BP1 (Galanty et al., 2009). Other DDR functions for SUMO E3 ligases are highlighted by the interaction between PIAS1 and SNM1A promoting ICL repair (Ishiai et al., 2004) and by sumoylation of tyrosyl DNA phosphodiesterase (TDP1) enhancing repair of single-strand DNA breaks (Hudson et al., 2012). In addition, while the SUMO protease SENP6 interacts with RPA70 in S phase, keeping RPA70 hyposumoylated, replication stress dissociates the complex, allowing accumulation of SUMO2/3-modified RPA70 that then promotes HR by recruiting RAD51 to damaged DNA (Dou et al., 2010). BLM, the RecQ family DNA helicase defective in human Bloom's syndrome, is also sumoylated, with mutations blocking its sumoylation causing higher DNA damage production during S phase, DNA damage hypersensitivity, and impaired RAD51 localization to sites of replication stalling (Ouyang et al., 2009).

Additional connections between ubiquitylation and sumoylation are highlighted by work on SUMO-targeted ubiquitin ligases (STUbLs), which include human RNF4, *S. pombe* Rfp1/2-Slx8, and *S. cerevisiae* Slx5-Slx8 (Prudden et al., 2007). STUbLs contain SIMs that bind sumoylations or SUMO-like domains on target proteins and then ubiquitylate such proteins, often leading to their proteasomal degradation. Although work has shown that *S. cerevisiae* Slx5-Slx4 and *S. pombe* Rfp1/2-Slx8 regulate HR, the mechanisms for this are not yet clear. By contrast, recent work revealed that RNF4 inactivation in human or chicken cells caused defective DSB repair by both HR and NHEJ (Galanty et al., 2012; Luo et al., 2012; Yin et al., 2012a). Moreover, RNF4 is recruited to DSB sites via interactions between its N-terminal SIMs and sumoylated DSB-response proteins such as 53BP1, MDC1, and RPA, where it mediates the accrual of ubiquitin adducts (Figure 6). While there are likely multiple, functional substrates for RNF4, major ones appear to be MDC1 and RPA, with RNF4 promoting their rates of turnover at DSB sites. For example, decreased RPA turnover caused by RNF4 depletion or mutation of RPA sumoylation sites leads to RPA being ineffectively replaced by RAD51, thereby impairing HR (Galanty et al., 2012). The fact that RNF4 promotes proteasome recruitment to DSB sites, together with proteasome subunit depletion phenocopying many aspects of RNF4 depletion, suggest that RNF4-mediated, SUMO-targeted

ubiquitylation of DDR factors leads to their recognition by the proteasome, thus triggering their localized turnover and coordinating progression through multiple stages of the DNA repair process. In this regard, the *S. pombe* VCP/p97 counterpart, Cdc48, binds to SUMO conjugates via a SIM in its cofactor Ufd1 (Nie et al., 2012). Because VCP also binds to ubiquitin, DNA repair might be promoted by VCP through binding STUbL targets that are comodified by both ubiquitin and SUMO. Given that *S. cerevisiae* Ufd1 also binds SUMO and because human VCP/p97 functions at DSB sites (see the preceding sections), connections between STUbL targets, VCP/p97 complexes, and the proteasome are likely to exist throughout the eukaryotic lineage.

Therapeutic Applications

DNA-damaging chemotherapies and radiotherapies are widely used and often-effective cancer treatments, with their efficacy reflecting the induction of lethal loads of DNA damage in cancer cells. Unfortunately, cancer cell mechanisms frequently lead to tolerance or repair of therapy-induced DNA lesions, incomplete eradication, and subsequent recurrence, while effects of DNA damage on normal tissues limit dosing and are associated with toxicities. The success of DNA-damaging agents as anticancer therapeutics seems to reflect cancer cells often proliferating more than most normal cells, through them experiencing higher DNA damage loads than normal cells and through them often being impaired in certain DDR components, making them particularly reliant on the ones they retain (Helleday et al., 2008; Jackson and Bartek, 2009). In addition to providing opportunities for better tailoring of DNA-damaging therapies to particular tumors, knowledge of DDR differences between cancers and normal cells also provides opportunities for killing cancers selectively via the concept of synthetic lethality, as first demonstrated by killing of HR-defective, *BRCA1/2*-deficient tumors by PARP1/2 inhibitors (Bryant et al., 2005; Farmer et al., 2005). Our growing knowledge of how ubiquitylation and sumoylation impact DDR processes might therefore yield new opportunities for cancer management. For example, the fact that inherited defects in ubiquitylation result in DDR deficiencies raises the prospect that such defects might arise somatically during cancer evolution, thereby providing new diagnostic and perhaps therapeutic opportunities. Furthermore, the array of proteins of the ubiquitin and SUMO systems—many of which are inherently druggable enzymes—provides a vast and presently largely untapped resource for new therapeutic targets. Notably, proteasome inhibition by bortezomib (Velcade) can sensitize cells to radiotherapy and DNA-damaging chemotherapies (Motegi et al., 2009), and recent work has indicated that it causes a HR-impaired state in multiple myeloma cells, thus causing hypersensitivity to PARP inhibitors and highlighting how combined use of such agents might have clinical potential (Neri et al., 2011). Development of agents targeting more DDR-specific components of the ubiquitin and SUMO systems might also have anticancer properties, and, being more focused on certain DDR events, these might have fewer toxicities than those caused by more general inhibitors of the ubiquitin-proteasome system. It is also possible that linkages of ubiquitin and SUMO with DNA repair can be exploited to provide better diagnostics and therapeutics for additional age-related diseases associated with DNA damage and/or DDR dysfunction.

Perspectives

The past few years have witnessed a rapid increase in our understanding of how ubiquitin and SUMO conjugation affect cellular DNA damage responses. This growth has been propelled by enhancements in our general understanding of the ubiquitin and SUMO systems, by the advent of new techniques and approaches, and by the large number of researchers now operating in this exciting area of research. In addition to identifying additional ubiquitin and SUMO system components that affect DNA repair and the associated events, another potentially exciting area for future study will be to assess whether and how other ubiquitin-like modifier proteins connect to the DDR. The major—and in our view the most exciting—challenge for future work, however, will be to explain precisely how ubiquitylation, sumoylation, and related events control DDR processes. As mentioned previously, in addition to being sometimes coupled as monomers, alone or in combination, ubiquitin and SUMO can also be conjugated through different linkages into a vast potential array of differing chain topologies with differing functional effects on target proteins. Emerging evidence points to such biological specificity often being achieved via modified proteins being bound by "reader" factors that combine ubiquitin/ubiquitin-like binding motifs and targeting sequences, thus allowing recognition of a particular ubiquitin/SUMO chain or linkage in the context of a specific protein substrate. The potential scope for biochemical and functional diversity in a "ubiquitin/SUMO/UBL code" is clearly enormous, and it is even larger when one considers that such modifications may also be recognized in conjunction with other protein modifications such as phosphorylation and poly(ADP) ribosylation. Other areas where we see the DDR contributing to our knowledge of ubiquitylation or sumoylation are in the area of VCP/p97 function and ubiquitin chain editing. Given its strength and breadth, it seems likely that the DDR research community will continue to discover new principles and paradigms for ubiquitylation and sumoylation, many of which should also apply to the wider array of cellular activities controlled by these two related posttranslational modifications.

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