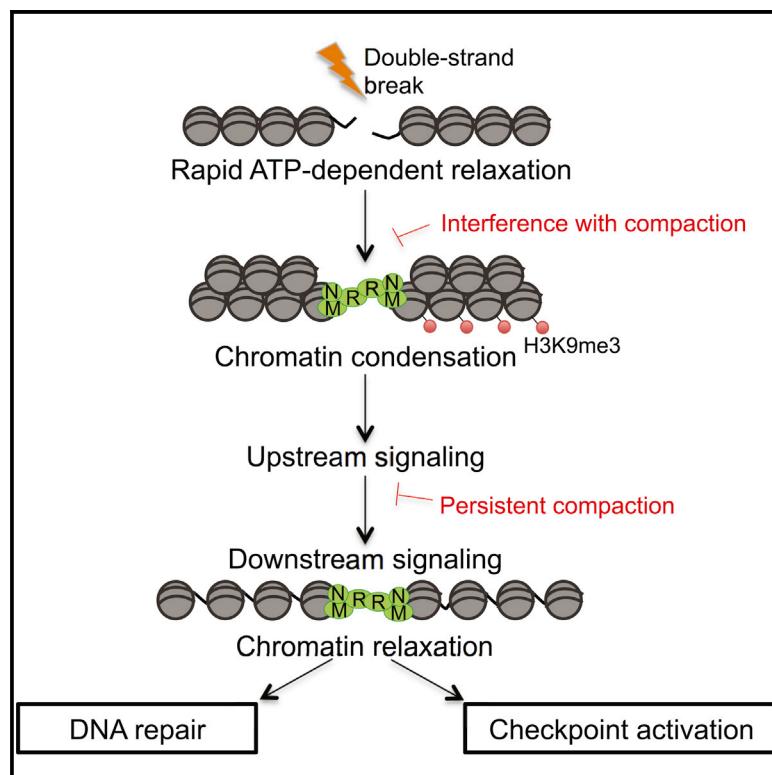


Activation of DNA Damage Response Signaling by Condensed Chromatin

Graphical Abstract



Authors

Rebecca C. Burgess, Bharat Burman,
Michael J. Kruhlak, Tom Misteli

Correspondence

mistelit@mail.nih.gov

In Brief

Relaxation of chromatin is important for checkpoint activation and DNA repair, but the role of chromatin condensation has been enigmatic. Burgess et al. show that chromatin condensation is an integral but transient part of the DNA damage response. Whereas condensed chromatin enhances upstream signaling, persistent condensation inhibits downstream repair and recovery.

Highlights

Dynamic compaction of chromatin is an integral step in the DNA damage response

Impairment of chromatin condensation attenuates signaling from a DNA break

Induced chromatin condensation can stimulate damage-independent upstream signaling

Persistent compaction boosts upstream signaling but inhibits repair and recovery



Activation of DNA Damage Response Signaling by Condensed Chromatin

Rebecca C. Burgess,¹ Bharat Burman,^{1,2} Michael J. Kruhlak,³ and Tom Misteli^{1,*}

¹National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA

²Program in Cell, Molecular and Developmental Biology, Sackler School of Biomedical Sciences, Tufts University, Boston, MA 02111, USA

³Experimental Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA

*Correspondence: mistelit@mail.nih.gov

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SUMMARY

The DNA damage response (DDR) occurs in the context of chromatin, and architectural features of chromatin have been implicated in DNA damage signaling and repair. Whereas a role of chromatin decondensation in the DDR is well established, we show here that chromatin condensation is integral to DDR signaling. We find that, in response to DNA damage chromatin regions transiently expand before undergoing extensive compaction. Using a protein-chromatin-tethering system to create defined chromatin domains, we show that interference with chromatin condensation results in failure to fully activate DDR. Conversely, forced induction of local chromatin condensation promotes ataxia telangiectasia mutated (ATM)- and ATR-dependent activation of upstream DDR signaling in a break-independent manner. Whereas persistent chromatin compaction enhanced upstream DDR signaling from irradiation-induced breaks, it reduced recovery and survival after damage. Our results demonstrate that chromatin condensation is sufficient for activation of DDR signaling and is an integral part of physiological DDR signaling.

INTRODUCTION

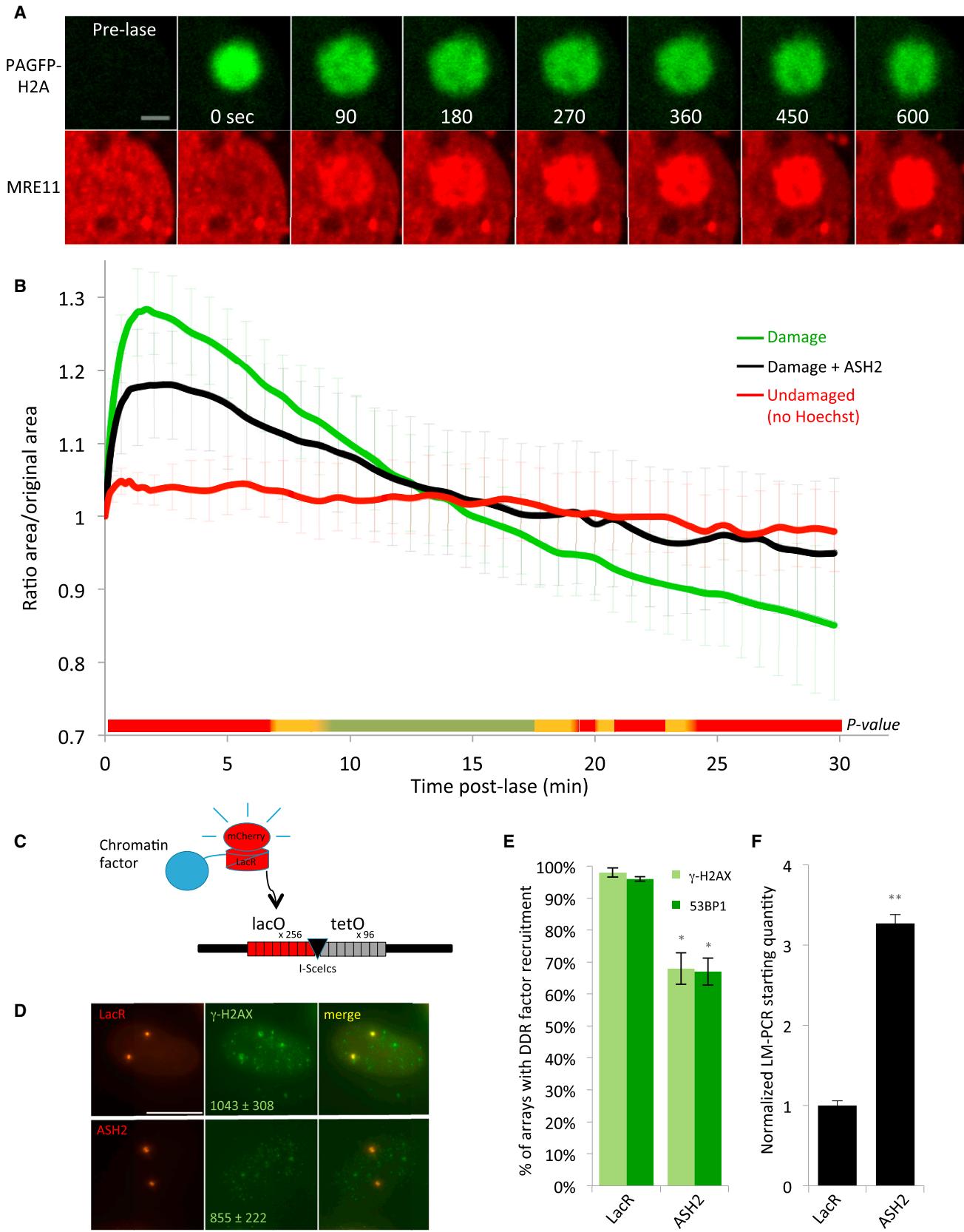
Upon sensing DNA damage, cells activate a complex signaling cascade termed the DNA damage response (DDR). The DDR triggers multiple cellular events including activation of DNA repair pathways, arrest of the cell cycle to allow time for repair, and, in certain cases, initiation of senescence or apoptosis programs (Ciccia and Elledge, 2010). The DDR functions within the context of chromatin and alterations in the structure of chromatin, as well as chromatin modifications, have been implicated in the activation and transduction of the DDR (Lukas et al., 2011; Price and D'Andrea, 2013; Shi and Oberdoerffer, 2012). The most prominent histone modification in the DDR is phosphorylation of the histone variant H2AX by the PIKK family of kinases, including ataxia telangiectasia mutated (ATM), ATR,

and DNA-PK, which generate large chromatin domains of phosphorylated H2AX (γ -H2AX) around double-strand breaks (DSBs) (Lee and Paull, 2005; Rogakou et al., 1999; Stiff et al., 2004). The γ -H2AX mark acts as a platform for hierarchical recruitment and retention of key DDR factors, including the mediator protein MDC1, promoting amplification of the DDR by further ATM activation and γ -H2AX spreading (Chapman and Jackson, 2008; Lou et al., 2006; Lukas et al., 2004; Stucki et al., 2005).

DDR activation leads to dynamic changes in chromatin structure, which contribute to the full-scale amplification and downstream functions of the DDR. Local chromatin decondensation, as well as histone reorganization and eviction, has been observed after experimental induction of DSBs in mammalian cells (Berkovich et al., 2007; Kruhlak et al., 2006; Ziv et al., 2006) and expedites downstream aspects of the DDR, including signaling through the CHK1 and CHK2 effector kinases and the engagement of repair pathways (Larsen et al., 2010; Murga et al., 2007; Murr et al., 2006; Polo et al., 2010; Smeenk et al., 2010).

A number of active chromatin processes to promote chromatin expansion for DNA repair have been proposed, including the phosphorylation and subsequent release of KAP-1, a binding partner of the structural heterochromatin protein HP1, as well as the relocation of DNA breaks to the periphery of cytologically detectable heterochromatin domains (Chiolo et al., 2011; Goodarzi et al., 2008; Jakob et al., 2011; Ziv et al., 2006). HP1 variants themselves are also phosphorylated and released from heterochromatin regions after induction of DSBs (Ayoub et al., 2008; Dinant and Luijsterburg, 2009).

Somewhat paradoxically, proteins that promote chromatin compaction, such as HP1, KAP-1, SPOC1, su(var)3-9 methyltransferase variant 1 (SUV3-9), PRDM2 methyltransferase, macro H2A, and histone deacetylases, have also been shown to be recruited to sites of DSBs (Ayoub et al., 2009; Ayrapetov et al., 2014; Baldeyron et al., 2011; Khurana et al., 2014; Luijsterburg et al., 2009; Miller et al., 2010; Mund et al., 2012; Noon et al., 2010; Polo et al., 2010; Smeenk et al., 2010; Zarebski et al., 2009). Recent work suggests that a transient repressive chromatin domain enriched in the histone H3 lysine 9 di- and trimethyl marks is established by PRDM2 and SUV3-9 methyltransferases being recruited to DNA damage sites (Ayrapetov et al., 2014; Khurana et al., 2014). In addition,



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the histone H3 lysine 9 trimethyl mark (H3K9me3) is known to stimulate binding and activation of the TIP60 acetyltransferase after DNA damage (Sun et al., 2009). TIP60 in turn acetylates ATM kinase, which promotes its activation (Sun et al., 2005). Interestingly, phosphorylation enhances the acetyltransferase activity of TIP60, and this modification can be induced by chromatin alterations, leading to ATM signaling independently of DNA breaks (Kaidi and Jackson, 2013). Here, we sought to directly test, in a controllable system, the role of chromatin condensation in the DDR-signaling cascade and its impact on cell survival.

RESULTS

Chromatin Condensation Is an Integral Part of the DDR

We sought to characterize changes in chromatin compaction in response to DNA damage. To this end, we used a previously characterized method based on a photoactivatable version of GFP (PAGFP) fused to the H2A core histone (Kruhlak et al., 2006). PAGFP can be activated simultaneously with laser micro-irradiation, allowing direct tracking of the chromatin dynamics of a damaged region (Kruhlak et al., 2006). In line with earlier observations (Kruhlak et al., 2006), upon local laser irradiation of a small spot of $\sim 4.5 \mu\text{m}$ in diameter, the damaged regions showed rapid expansion, reaching a maximum at about 1.5 min postirradiation, with MRE11 recruitment detectable immediately (Figures 1A, 1B, and S1A). This expansion was followed by an extended linear recompaction phase, reaching predamage levels by 15 min, followed by hypercondensation beyond the predamage baseline level by 20–30 min postdamage (Figure 1B). No chromatin changes were observed in undamaged control cells (no Hoechst sensitization; 355 nm/405 nm laser irradiation; Figure 1B). Damage-induced chromatin changes were dampened in both the expansion and compaction phases after overexpression of the Set1/Ash2 methyltransferase ASH2L (Figure 1B), which globally increases the H3 lysine 4 methyl mark that is implicated in transcriptional activation and mediates chromatin expansion through recruitment of chromatin modifiers (Boyle et al., 2008; Chambeyron and Bickmore, 2004; Birney et al., 2007; Ling et al., 2010; Luco et al., 2010; Santos-Rosa et al., 2002; Shimada et al., 2006). In agreement with recent findings (Khurana et al., 2014), we conclude that the DNA damage

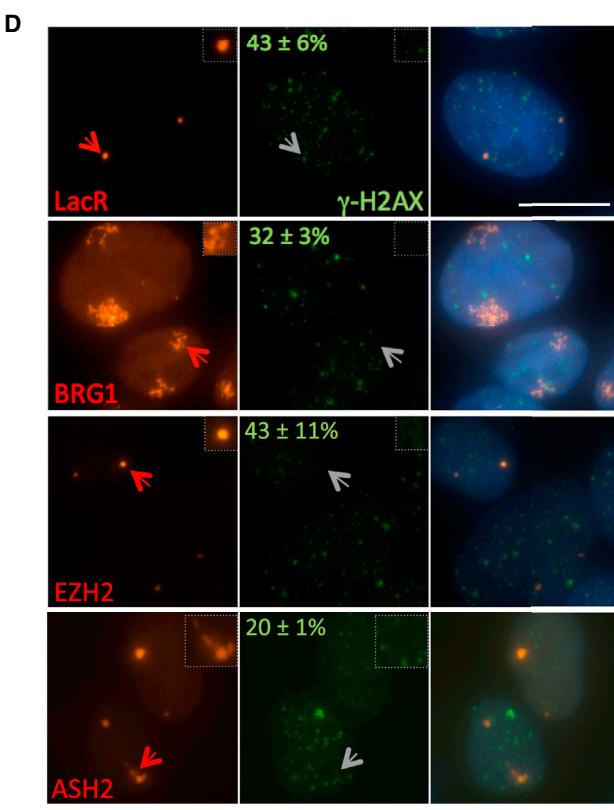
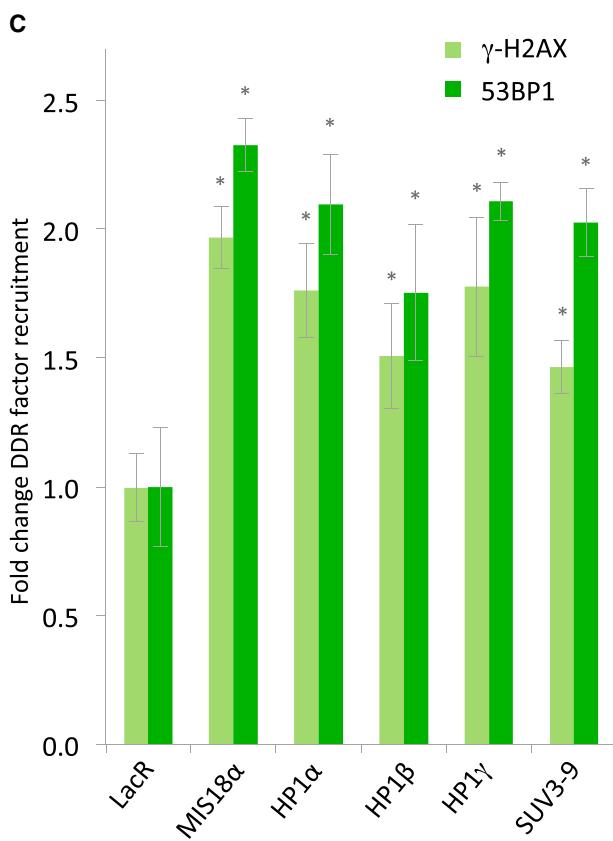
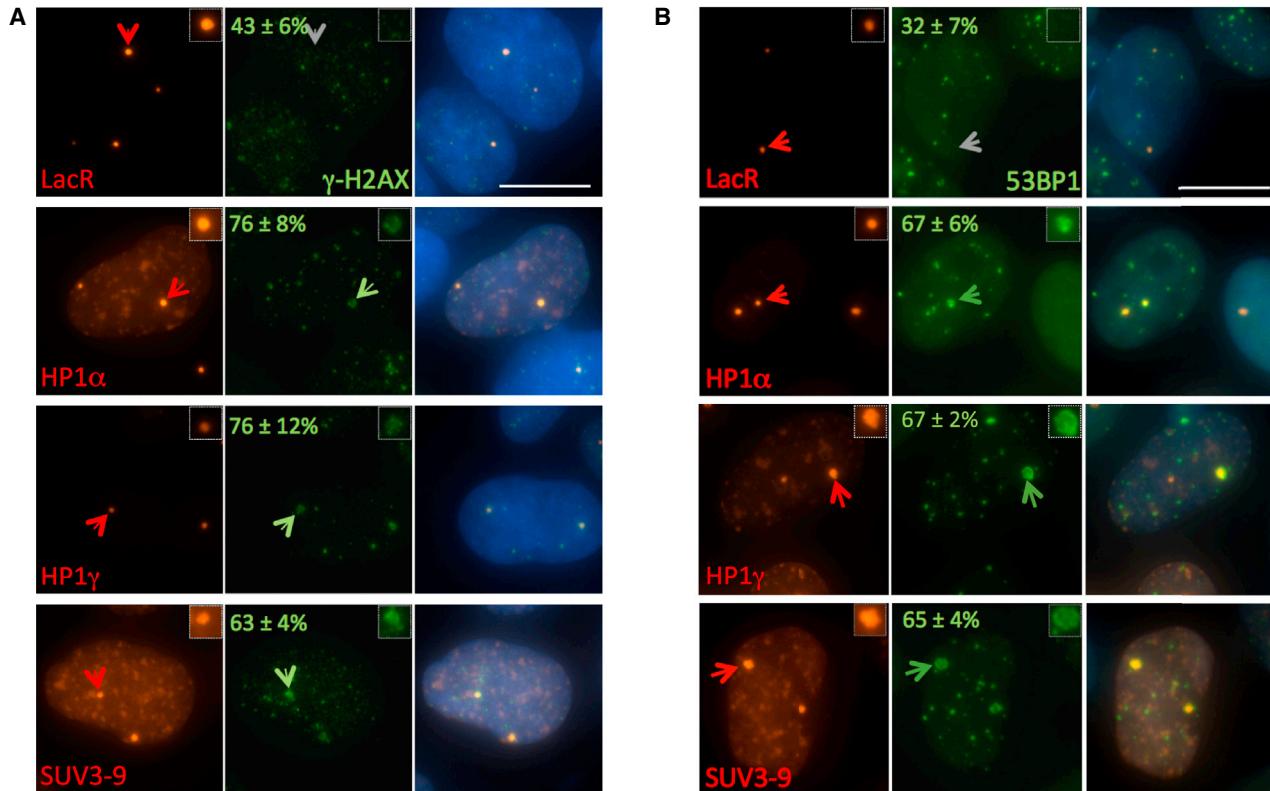
response involves initial expansion of chromatin followed by a phase of chromatin condensation.

To directly probe the role of chromatin condensation in the DDR, we used a previously characterized Lac-repressor/operator-tethering system to create chromatin domains with defined properties (Soutoglou and Misteli, 2008). The system consists of ~ 10 kb tandem arrays of the Lac operator (lacO) sequence adjacent to an I-SceI endonuclease site stably integrated into human U2OS cells at two different chromosomal locations (Figure 1C). Defined chromatin domains can be generated by virtue of tethering fusion proteins between chromatin modifiers and the Lac repressor (LacR) protein, which binds with high affinity to the lacO regions (Soutoglou and Misteli, 2008). Having observed an inhibitory effect of ASH2 on chromatin compaction after DNA damage (Figure 1B), we sought to probe how interference with chromatin condensation affects DDR signaling. To this end, we tethered ASH2-LacR tagged with mCherry to the lacO arrays (Figure 1D). As expected, tethering of ASH2 led to enrichment of the H3K4me3 mark at the array, but not at the cyclophilin A 3' UTR site (Figure S1B). As shown previously for the VP16 trans-activator (Tumbar et al., 1999), tethering of ASH2 led to visible expansion of array sites, demonstrating local chromatin expansion (Figure S1C). After establishment of the ASH2-expanded chromatin domain in cells, we transfected a glucocorticoid-receptor-I-SceI endonuclease fusion (GR-I-SceI) into cells to synchronously and continuously induce DSB formation by addition of a synthetic GR ligand (dexamethasone), which stimulates translocation of the GR-I-SceI fusion into the nucleus (Soutoglou et al., 2007). Upon induction of DSBs in control cells expressing LacR-mCherry, γ -H2AX and 53BP1 accumulated at the I-SceI-containing arrays in more than 90% of cells within 20 min (Figures 1D and 1E). In contrast, the number of arrays that showed detectable accumulation of γ -H2AX and 53BP1 was reduced by $\sim 35\%$ in cells with expanded chromatin arrays due to tethering of ASH2 (Figures 1D and 1E). In addition, the γ -H2AX signal at damaged arrays was slightly weaker in ASH2-expressing cells (median intensity: 855 ± 222) than in control cells ($1,043 \pm 308$; $p < 0.05$; Figure 1D). Importantly, the reductions in γ -H2AX and 53BP1 were not due to lower levels of DSB induction in ASH2-expressing cells. On the contrary, ASH2-expressing cells showed an ~ 3 -fold higher level of DSBs compared to LacR-expressing control cells as determined by ligation-mediated PCR to directly quantify

Figure 1. Chromatin Undergoes Rapid Expansion and Compaction after DNA Damage, and Interference with These Chromatin Changes Attenuates DDR Signaling

- (A) Snapshots of a damaged chromatin region and recruitment of MRE11 at indicated time points. PAGFP-H2A, green; mCherry-tagged MRE11, red. The scale bar represents $3 \mu\text{m}$.
- (B) Average area of damaged chromatin regions in vector control (green line) versus ASH2-overexpressing cells (black line) over time; an undamaged chromatin region is shown in red (no Hoechst sensitization). Error bars depict SD at each point. p values of ASH2 versus control are shown in heatmap below. Red, $p < 0.01$; orange, $p < 0.05$; green, not significant. $n > 15$ regions for each point.
- (C) Schematic of chromatin-protein-tethering system: 256 copies of the lac operator (lacO) and 96 copies of tet (tetO) flank an I-SceI cut site (I-SceIcs). Lac repressor fusions to either mCherry alone (LacR) or to chromatin proteins bind to the lac operator arrays after transient expression.
- (D) Maximum intensity projections of LacR- or ASH2-tethered arrays (red) stained for γ -H2AX (green) after DSB induction by CFP-GR-I-SceI. The scale bar represents $5 \mu\text{m}$. Values show median integrated intensity of γ -H2AX at arrays \pm median absolute deviation.
- (E) Percentage of LacR- or ASH2-tethered arrays with γ -H2AX or 53BP1 enrichment 20 min after DSB induction. Columns depict mean and error bars, SD. $n \geq 100$ for each condition. * $p < 0.05$.
- (F) Ligation-mediated quantitative PCR detecting the quantity of DSBs in I-SceI-induced cells. Shown is the average \pm SD of two independent experiments, each performed in triplicate. ** $p < 0.001$.

See also Figure S1.



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the total number of DSBs after 20 min of steady-state break induction (Figure 1F; Soutoglou et al., 2007). Our finding of reduced γ-H2AX and 53BP1 recruitment to DSBs under conditions of persistent chromatin decondensation, despite higher levels of induced DSBs, points to a role of chromatin condensation in DDR signaling.

Chromatin Condensation Triggers DDR Signaling

To directly assess the effect of chromatin condensation on the DDR, the heterochromatin structural protein HP1 or the H3K9 methyltransferase SUV3-9, which creates HP1 binding sites, were tethered to the lacO repeats. HP1 tethering to lacO has previously been shown to induce chromatin compaction and silencing as indicated by morphological and accessibility assays, as well as gene expression analysis (Danzer and Wallrath, 2004; Li et al., 2003; Verschure et al., 2005). Consistent with condensation, tethering of these repressive chromatin proteins led to more-compact arrays (Figure S1D). As expected, tethering of HP1 α , HP1 γ , or SUV3-9 led to enrichment of H3K9me3 at the array, as judged by chromatin immunoprecipitation (ChIP) and immunofluorescence against H3K9me3 (Figures S2A and S2B). In line with chromatin compaction, tethering of heterochromatin factors also decreased accessibility to nuclease cutting (\log_2 starting quantity = -0.1 to -0.4), compared to the lac array alone (Figure S2C). Chromatin accessibility of distant silenced (*HBB*) or expressed (*GAPDH*) chromosomal loci was unaffected by expression and tethering of chromatin factors (Figure S2D). Likewise, no changes in the overall levels or distribution of the H3K9me3 heterochromatin mark were detected by ChIP or immunostaining at unrelated loci (Figures S2A and S2B), further confirming the site-specific, rather than global, effect of locally tethering chromatin compaction factors.

Tethering of chromatin compaction factors allowed us to directly assess the effect of chromatin condensation on DDR signaling. We found that tethering of HP1 α , - β , or - γ was sufficient to trigger DDR signaling, even in the absence of DNA damage (Figure 2A). Upon tethering of HP1 α , - β , or - γ , ~75% of arrays showed accumulation of phospho-H2AX and ~65% showed recruitment of 53BP1 compared to 30%–35% in LacR controls (Figures 2A–2C; $p < 0.05$). Similarly, tethering of SUV3-9 had a similar effect and led to a 2-fold increase in accumulation of γ-H2AX (63% of arrays) and recruitment of 53BP1 (65% of arrays) compared to LacR alone (Figures 2A–2C; $p < 0.05$). As a positive control for DDR activation, LacR-MIS18 α was used, which creates DNA breaks when tethered to the array (Figure 2C; see Figure 3A below). The mCherry-LacR-HP1 fusions and mCherry-LacR-SUV3-9 localized most intensely to

arrays but, as expected, also appeared at sites of endogenous heterochromatin (Figures 2A, 2B, and S2B; Verschure et al., 2005). However, DDR factors were not corecruited to heterochromatin domains, indicating specificity of the DDR trigger to the tethering array (Figure 2A). This DDR activation was a specific response to chromatin condensation and did not reflect a response to arbitrary changes in chromatin structure because no DDR activation was observed upon chromatin decondensation in the absence of I-SceI cutting (Figure 2D). Tethering of chromatin expansion factors, such as ASH2, VP16, or the SWI/SNF chromatin remodeler BRG1, visibly expanded the lac arrays but, in contrast to condensed arrays, did not recruit 53BP1 or induce γ-H2AX (Figure 2D; data not shown). Furthermore, tethering of EZH2, a polycomb family protein involved in facultative heterochromatin formation, did not recruit DDR factors (Figure 2D), suggesting that activation of the DDR by chromatin condensation involves specific features of heterochromatin.

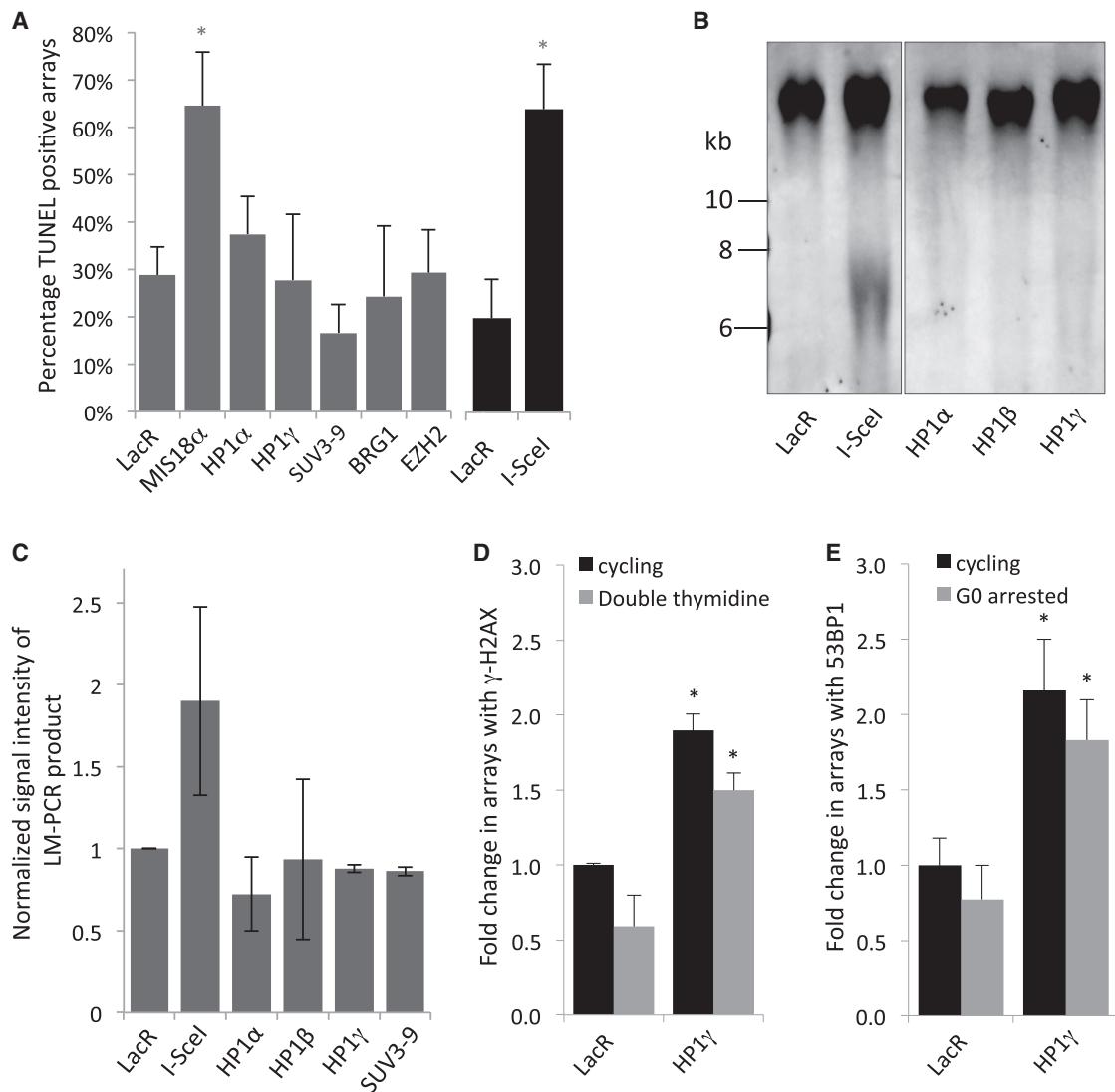
Several lines of evidence rule out that DDR signaling upon array-localized chromatin condensation is the result of DNA breaks induced by tethering. No labeling by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was detected (Figure 3A); Southern blotting using probes to the Lac array did not reveal tethering-induced DSBs in the arrays (Figure 3B), and no breaks were detected by ligation-mediated PCR, which sensitively amplifies DSBs near the lacO integration site (Soutoglou and Misteli, 2008; Figure 3C). Furthermore, limiting the expression of tethering constructs to 20 hr after transient transfection prevented potential replication defects caused by extended tethering of fusion proteins to the Lac array (Beuzer et al., 2014; Jacome and Fernandez-Capetillo, 2011). In line with this, DDR activation by chromatin condensation was not a cell cycle effect or due to stalled replication, because it was similarly observed in cells arrested either at G1/S or in G0 (Figures 3D and 3E). Taken together, these data suggest that condensed chromatin is sufficient to stimulate DDR signaling.

Condensed Chromatin Promotes Activation of Upstream Parts of the DDR

To assess whether chromatin condensation leads to recruitment of other DNA repair factors, we measured the accumulation of DDR components at the condensed array (Figure 4A). In addition to γ-H2AX and 53BP1, the MDC1 mediator protein and NBS1, a component of the MRE11-RAD50-NBS1 (MRN) complex, were efficiently recruited to locally compacted chromatin domains (Figures 2C and 4A). Their extent of recruitment was similar

Figure 2. Compacted Chromatin Triggers DDR Signaling

- (A) Maximum intensity projections of cells transfected with indicated mCherry-LacR constructs (red) stained with anti-γ-H2AX (green) and DAPI (blue). Green arrows indicate arrays enriched in γ-H2AX that are magnified 2 \times in the inset image. Gray arrows indicate arrays without significant γ-H2AX. The scale bar represents 5 μ m. Percentages \pm SD of arrays staining positive for γ-H2AX are shown in the top center of each panel.
 - (B) Images of cells as in (A) but stained with anti-53BP1 (green)
 - (C) Quantification of γ-H2AX and 53BP1 colocalization frequency at the arrays. Values represent fold change in averages \pm SD from three experiments ($n \geq 300$ for each condition). * $p < 0.05$ compared to LacR alone.
 - (D) Images of cells as in (A) but expressing indicated chromatin-expansion factors or facultative heterochromatin proteins fused to the LacR protein. The scale bar represents 5 μ m.
- See also Figure S2.

**Figure 3. DDR Signaling from Arrays Is Not Due to Array Breakage or Replication Defects**

(A) Quantification of arrays showing positive TUNEL signals. I-SceI-transfected cells and MIS18 α arrays provide positive controls for DNA end detection. Values represent averages \pm SD from at least three experiments. *p < 0.05 compared to LacR alone; n > 200 for each.

(B) Southern blot of genomic DNA isolated from indicated tethering conditions, using a probe to the lac array. I-SceI-transfected cells provide positive control for breaks; unrelated lanes from the blot between I-SceI and HP1 α omitted for simplicity.

(C) Ligation-mediated PCR assay detecting damage within the lac array. I-SceI used as a positive control for breaks. Normalized signal intensity of PCR reactions are depicted by averages \pm SD from two independent trials.

(D and E) Cells (D) prearrested in G1 by double thymidine block or (E) serum starved prior to transfection with LacR alone- or HP1 γ -tethering constructs. Values represent averages \pm SD of γ -H2AX or 53BP1 recruitment measured in cyclin A- or Ki67-negative cells, respectively, from two to five independent experiments. n > 150 per condition. *p < 0.05 compared to cycling cells.

See also Figure S3.

with 65%–75% of arrays exhibiting recruitment, representing a 2-fold increase over control cells (Figure 4A). Accumulation of these factors was not due to direct interaction with HP1 because their recruitment was inhibited under conditions where HP1 was tethered to a decondensed lac array (Figures S3A–S3D). This cotethering experiment did not reflect outcompetition of HP1 binding by the activators, because both fusions were able to bind the array without much competition as evident by visible deconden-

sation with a robust HP1 signal. In addition, increasing amounts of LacR did not inhibit the HP1 effect, consistent with lac operators being incompletely occupied at any given time (Figures S3E and S3F). Although we cannot fully exclude the possibility that cotethering of activators leads to enhanced clearance of DDR factors, we think this unlikely because we observe heightened levels of γ -H2AX at damaged arrays expanded by BRG1 (R.C.B. and T.M., unpublished data). We take these data to

suggest that HP1 does not act as a platform for DDR factor recruitment but that condensed chromatin structures induce upstream DDR signaling.

The activation and amplification of the DDR involves several overlapping kinase activities, particularly, ATM, ATR, and DNA-PK (Burma et al., 2001; Stiff et al., 2004; Ward and Chen, 2001). Active ATM monomers are exposed by their phosphorylation at serine 1981 (Bakkenist and Kastan, 2003), and tethering of SUV3-9, HP1 α , - β , or - γ resulted in robust accumulation of phosphorylated ATM at the array (Figure 4B). To distinguish the contributions of ATM and ATR in chromatin-activated DDR signaling, we performed small interfering RNA (siRNA) knockdowns of ATM or ATR as well as dual knockdown of both kinases simultaneously (Figures S4A and S4B). DDR activation was only significantly inhibited when both ATM and ATR were depleted (Figure 4C; $p < 0.05$). Experiments with specific kinase inhibitors confirmed these results. Treatment of cells containing LacR-HP1 γ with specific inhibitors to both ATM and ATR or to all three kinases led to a significant decrease in the frequency of γ -H2AX at the HP1-tethered arrays to near background levels (Figure 4D; $p < 0.01$). Inhibitors of ATR or ATM alone led to more-modest decreases in γ -H2AX signaling ($p < 0.05$), whereas an inhibitor specific to DNA-PK did not significantly curtail phosphorylation of H2AX (Figure 4D). Altogether, these observations demonstrate that ATM and ATR are jointly involved in the chromatin-induced DDR signaling.

ATM signaling normally activates CHK2 kinase and downstream targets SMC1 and p53 (Hirao et al., 2000; Matsuoka et al., 2000; Yazdi et al., 2002). However, these factors were not activated by tethering of HP1 α , - β , or - γ (Figure 4E), suggesting that key effectors of the conventional DDR are not globally activated by condensed chromatin domains. Lack of activation of downstream cell-cycle checkpoints by chromatin condensation was confirmed by cell-level analysis of cyclin A immunostaining (Figure 4F). We also found no difference in the cell-cycle profiles of large populations using cell sorting, with neocarzinostatin treatment as a positive control (Figure 4G). We conclude that chromatin condensation contributes to restricted activation of upstream components of the DDR, but not downstream effectors.

Condensed Chromatin Activates Upstream Components of DDR Signaling in Mitotic Cells

Mitotic chromosomes are an extreme case of naturally occurring condensed chromatin. If condensed chromatin contributes to DDR signaling and is sufficient to activate parts of the DDR, a prediction is that mitotic chromosomes should trigger upstream DDR signaling. Consistent with our findings on induced condensed chromatin domains, we detect accumulation of γ -H2AX on mitotic chromosomes, as previously observed by others (Ichijima et al., 2005; McManus and Hendzel, 2005). The intensity of γ -H2AX foci on mitotic chromosomes was increased about 3-fold compared to interphase levels as judged by integrated focus density measurements (Figure 5A). In addition, mitotic chromosomes were decorated by MDC1 foci, which colocalized, albeit weakly in some cases, with γ -H2AX foci, whereas 53BP1 was excluded from mitotic γ -H2AX foci, as previously reported (Figure 5B; Giunta et al., 2010; Nakamura et al.,

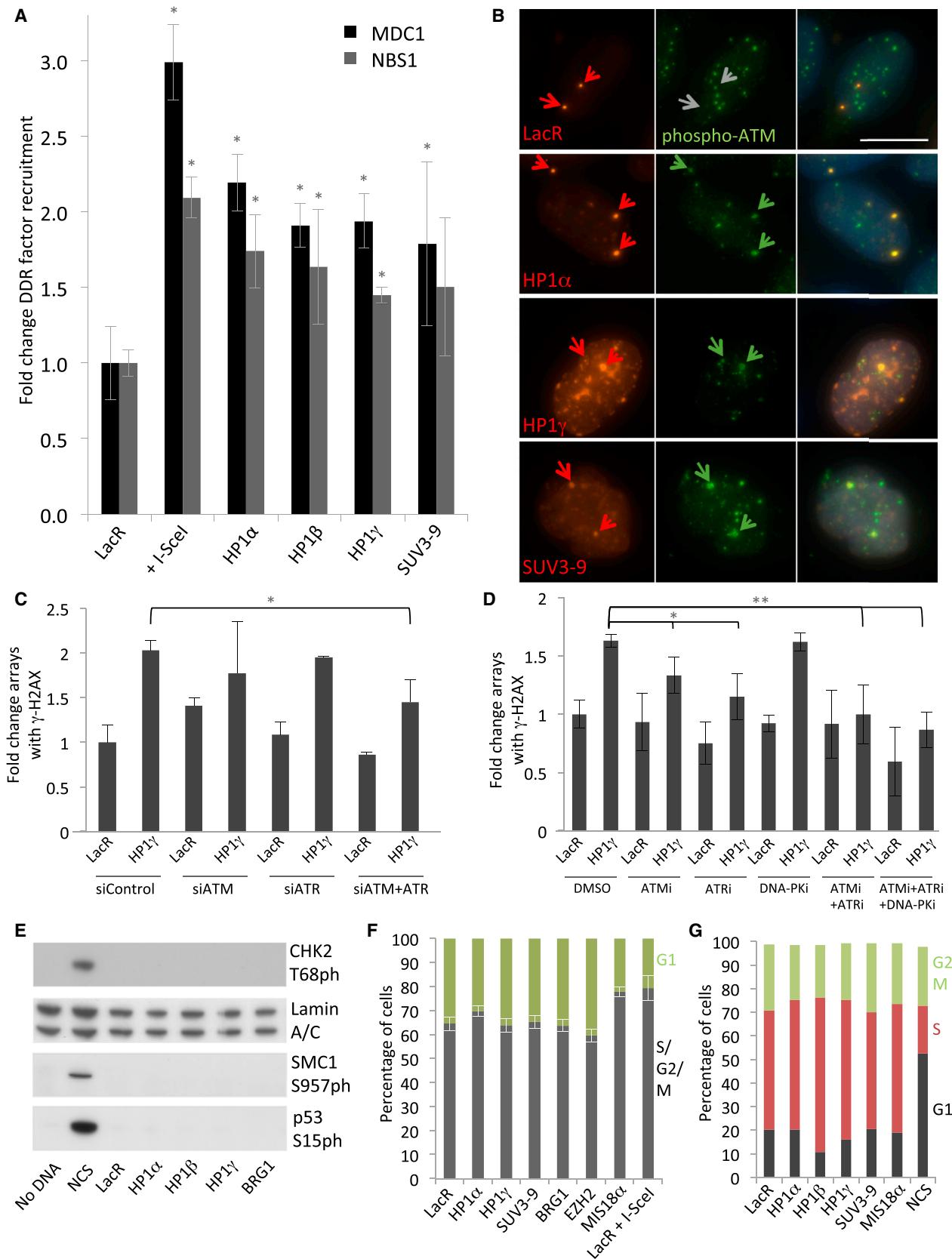
2010; Oricchio et al., 2006). As expected, no free DNA ends were detected on mitotic chromosomes by TUNEL (Figure S5), suggesting that the constitutive mitotic DDR signaling occurred in the absence of DNA damage. Similarly, an increase in γ -H2AX foci in the absence of detectable DNA breaks was observed upon premature chromosome condensation induced by the phosphatase inhibitor calyculin A (Coco-Martin and Begg, 1997; Huang et al., 2006; Figures 5C and S5). Both ATM and ATR contributed to the mitotic chromatin-induced DDR, as treatment with caffeine diminished pannuclear γ -H2AX levels further than ATM inhibition alone (Figure 5D). These findings are in line with the observation of mitotic activation of ATM and recruitment of the MRN complex and MDC1 to mitotic chromosomes in the absence of activation of downstream portions of the pathway (Giunta et al., 2010).

Condensed Chromatin Boosts Upstream DDR Signaling but Is Detrimental to Downstream Repair and Recovery

Given that condensed chromatin regions can generate local upstream DDR signaling and the observation that damaged chromatin regions undergo compaction, we asked how these dynamics affect the signaling and repair of DSBs. To this end, we globally compacted chromatin by overexpressing the SUV3-9 methyltransferase and then produced DSBs throughout the genome with 5 Gy of γ irradiation. Compared to controls, SUV3-9-overexpressing cells showed a greater than 2-fold increase in NBS1 phosphorylation and increased γ -H2AX, suggesting enhanced early ATM signaling (Figures 6A, 6B, and S6A; $p < 0.01$). On the other hand, phosphorylation of CHK2 decreased by about 2-fold, representing a reduced ability of DSBs in compacted chromatin environments to signal to downstream effectors (Figures 6C and 6D; $p < 0.05$). To finally ask whether persistent chromatin compaction affects the recovery and survival of cells from DNA damage, we performed clonogenic assays. Survival of SUV3-9-expressing cells was markedly decreased compared to control cells (Figure 6E). From this, we conclude that, whereas chromatin compaction enhances upstream signaling, its interference with decondensation negatively impacts repair and recovery from DNA damage.

DISCUSSION

We provide evidence here that chromatin condensation contributes to DDR signaling. Based on our morphological observations and in line with recent findings by others (Ayrapetov et al., 2014; Khurana et al., 2014), we propose that condensation of chromatin is an integral step in the damage response. Using chromatin-tethering approaches to probe the functional effects of condensed chromatin during DDR, we find that condensed chromatin domains are sufficient to trigger ATM/ATR-dependent signaling and activate upstream, but not downstream, components of the DDR cascade. Conversely, interference with chromatin compaction at the site of DNA damage attenuates DDR signaling. These observations suggest that changes in chromatin structure are not just bystanders but actively contribute to DDR activation. Fitting with a role of highly condensed chromatin as a trigger for DDR signaling, we find upstream parts of the DDR activated in naturally occurring, highly



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condensed mitotic chromosomes. Whereas condensation augments upstream signaling, it renders damage refractory to repair and represses the recovery of cells from DNA damage, suggesting the need for a dynamic exchange in chromatin structure for an efficient DDR in physiological settings. Combined with recent observations by others (Ayrapetov et al., 2014; Khurana et al., 2014), these findings highlight the need for coordinated chromatin decondensation as well as condensation events for efficient activation of the DDR in a physiological setting.

A role of chromatin structure as a trigger of upstream DDR signaling is consistent with several previously reported circumstantial observations. Numerous studies have detected DDR activation under chromatin-altering conditions (Bakkenist and Kastan, 2003; Bencokova et al., 2009; Hunt et al., 2007). Hypotonic conditions, chromatin factor depletion, and treatments with trichostatin A or chloroquine that result in chromatin changes, but not DNA damage, have been reported to activate ATM (Bakkenist and Kastan, 2003; Kaidi and Jackson, 2013). In addition, damage-independent ATM activation has been reported in cells where senescence or replication stress has been induced, with concomitant formation of repressive chromatin (Olcina et al., 2013; Pospelova et al., 2009). Even in unperturbed cells, repressive chromatin domains such as subtelomeric and centromeric regions show enrichment for γ -H2AX, as revealed by several genome-wide studies (Kitada et al., 2011; Lee et al., 2014; Seo et al., 2012; Szilard et al., 2010). DDR activation has also been suggested in condensed chromosomes progressing through mitosis. Mitotically condensed and prematurely condensed chromatin have previously been shown to activate ATM with a corresponding increase in γ -H2AX foci and, again, an absence of detectable DNA damage (Huang et al., 2006; Ichijima et al., 2005; McManus and Hendzel, 2005; Oricchio et al., 2006). Furthermore, in cells lacking the mitotic chromokinesin motor protein KIF4, abnormally highly condensed chromosomes are heavily decorated by γ -H2AX yet progress through mitosis, indicating the absence of extensive DNA lesions or checkpoint signaling (Mazumdar et al., 2006). Finally, recent work has raised the intriguing notion that ATR kinase activity is triggered by mechanical stimuli resulting from condensation in mitotic prophase and functions to modulate chromatin engagement with the nuclear envelope, preventing aberrant topological configurations (Kumar et al., 2014).

This study and recently published work shows that chromatin compaction is an integral part of the generic DNA damage response (Ayrapetov et al., 2014; Khurana et al., 2014). We extend these findings by demonstrating that chromatin compac-

tion is sufficient to trigger the upstream activation of the DDR independently of the DNA lesion. This conclusion is in line with earlier tethering experiments demonstrating that a DNA lesion is not an obligatory part of the DDR machinery and DDR signaling can occur in its absence, allowing for the possibility of DDR initiation by stimuli other than DNA damage, such as chromatin structure (Bonilla et al., 2008; Soutoglou and Misteli, 2008). We suggest that experimentally condensed chromatin provides the structural and molecular environment mimicking a DDR amplification step, leading to constrained ATM and ATR signaling without providing the full context of downstream DDR. A signaling function for chromatin may aid in amplification of the DDR; it has previously been noted that rapid activation of ATM kinase after a DSB occurs within seconds after irradiation and the majority of the relatively large cellular pool of ATM is activated by only a few strand breaks (Bakkenist and Kastan, 2003). This rapid and extensive activation of the DDR suggests the existence of cellular mechanisms that sense the damage signal with exquisite efficiency. Compaction of chromatin structure around the break site may represent a potent means to augment the signaling domain generated by a single DSB (Ayrapetov et al., 2014; Bakkenist and Kastan, 2003).

The establishment of a compact chromatin domain for enhancing DDR signaling is complementary to the observation that chromatin relaxation is required for amplification and activation of DDR effector pathways, and they likely occur in a dynamic exchange (Burgess et al., 2012; Soria et al., 2012). In a recent study, SUV3-9 was shown to be recruited to DSBs, establishing the repressive H3K9me3 histone mark at sites of damage, which captures HP1, KAP-1, and further SUV3-9, propagating the heterochromatin domain for tens of kb surrounding a DSB (Ayrapetov et al., 2014). Formation of a H3K9me3-repressive mark creates binding sites for the TIP60 acetyltransferase, which then contributes to the amplification of ATM activity (Ayrapetov et al., 2014; Sun et al., 2005, 2009). In agreement, we find that the establishment of a H3K9 methyl domain can promote signaling, whereas decompaction of chromatin by overexpression or tethering of the ASH2 H3K4 methyltransferase dampens the DDR and local dynamics of chromatin after damage. Later, ATM signaling leads to KAP-1 phosphorylation and release of the KAP-1/HP1/SUV3-9 and CHD3 complexes, promoting the relaxation of the chromatin that is essential to downstream signaling and repair (Ayrapetov et al., 2014; Goodarzi et al., 2008, 2011). Impairing this relaxation by persistent compaction decreased the recovery of cells from damage in a clonogenic survival assays, consistent with a requirement for dynamic

Figure 4. Activation of Upstream DDR Signaling by Condensed Chromatin

- (A) Recruitment of MDC1 and NBS1 to condensed arrays, quantified as in Figure 2C. Values represent average fold change \pm SD from three independent experiments ($n \geq 200$ for each condition). * $p < 0.05$ compared to LacR alone.
- (B) Maximum intensity projections of phospho-ATM (S1981) immunostaining at LacR, HP1-, or SUV3-9 arrays. Green arrows, arrays enriched in phospho-ATM; gray arrows, no enrichment. The scale bar represents 5 μ m.
- (C) γ -H2AX formation in condensed chromatin following siRNA depletion of ATM and/or ATR, depicted as average fold change to control siRNA (siControl) transfected with mCherry-LacR \pm SD from two experiments ($n = 40-150$); * $p < 0.05$. Knockdown is shown in Figure S4.
- (D) DNA-PK, ATM, and ATR were inhibited with KU55933 (ATMi), VE-821 (ATRi), or NU7441 (DNA-PKi) after tethering. Average \pm SD of γ -H2AX recruitment compared to DMSO-treated cells transfected with LacR from three independent trials. $n > 175$ per condition; * $p < 0.05$; ** $p < 0.01$.
- (E) Immunoblot analysis of activated DDR factors detected by phospho-specific antibodies. Loading control, lamin A/C.
- (F and G) Cyclin A staining (F) and FACS cell cycle (G) profiling of cell populations with tethered chromatin factors. Shown is average percentage of cells positive (S/G2, M phases), or negative (G1) for cyclin A staining or as determined by DNA content. (F) Error bars represent SEM.

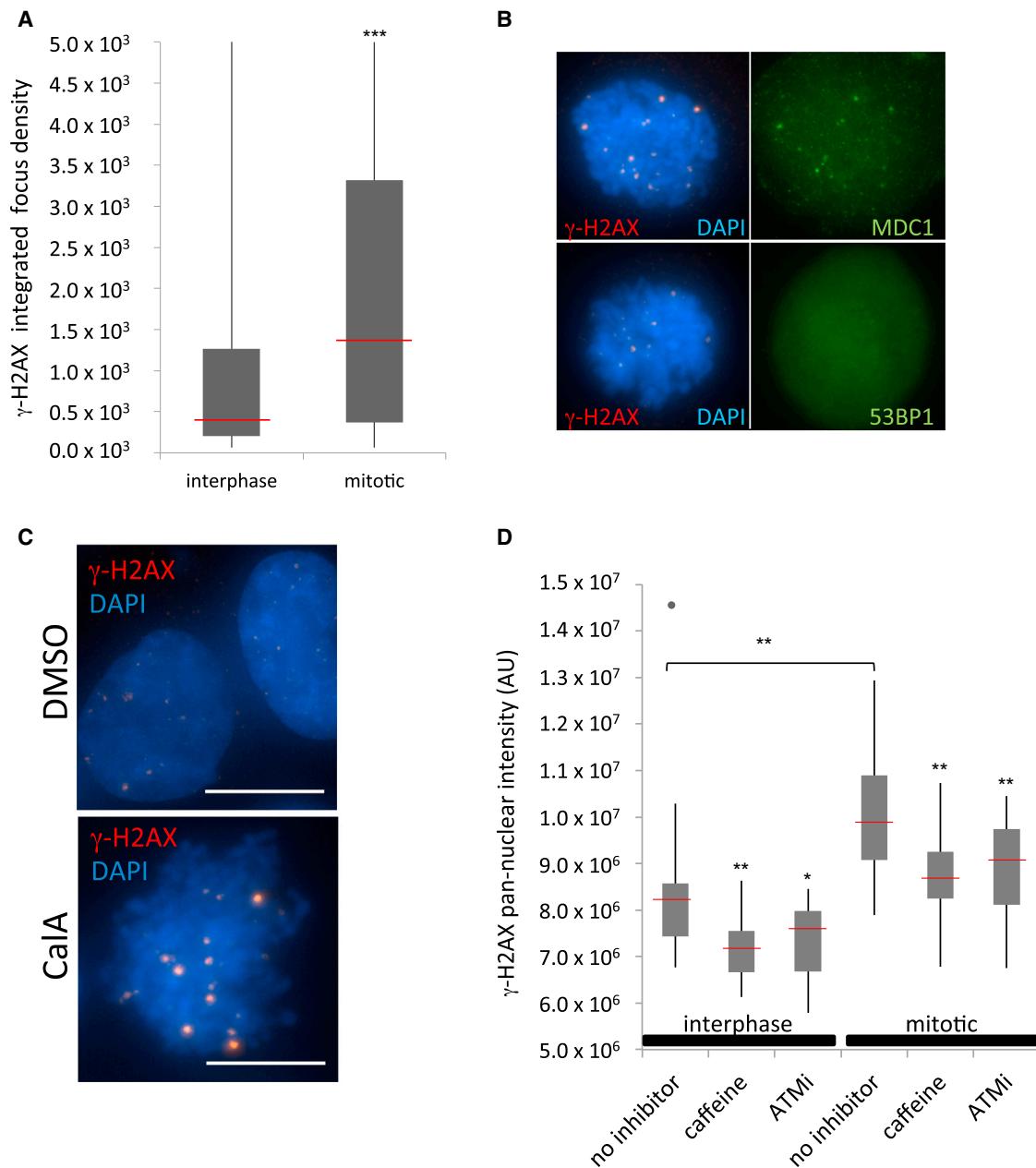


Figure 5. Mitotic Chromosome Condensation Activates Chromatin-Induced DDR

(A) γ -H2AX foci quantified by integrated density measurements. Box: quartiles 1–3; whiskers: value range; red bar: median values as follows: 487 (interphase) and 1,317 (mitosis). ***p < 0.001; n > 300 foci for each.

(B) Mitotic cells were harvested by shake off, and stained for γ -H2AX (red) and either MDC1 or 53BP1 (green). Shown are maximum intensity projections with DAPI overlay (blue).

(C) Increased γ -H2AX foci in cells treated with 50 nM CalA for 60 min, fixed, and immunostained for γ -H2AX (red) and DAPI (blue). The scale bar represents 10 μ m.

(D) Total nuclear intensity of γ -H2AX from cells in indicated treatments. Box and whiskers as in (A). One single outlier (>1.5 times outside the interquartile range) is indicated by a gray dot, and the red bars indicate median values. *p < 0.05; **p < 0.01. AU, arbitrary units.

See also Figure S5.

chromatin changes during DDR. Establishing the repressive chromatin domain is also important for the outcome of the DDR, as SUV3-9, PRDM2, or HP1 depletion impairs survival after DNA damage and may shift repair pathway choice (Ayrapetov

et al., 2014; Baldeyron et al., 2011; Khurana et al., 2014; Soria and Almouzni, 2013). This suggests that perturbing the dynamics of chromatin condensation and subsequent decondensation may be unfavorable to DDR. In addition, and not mutually

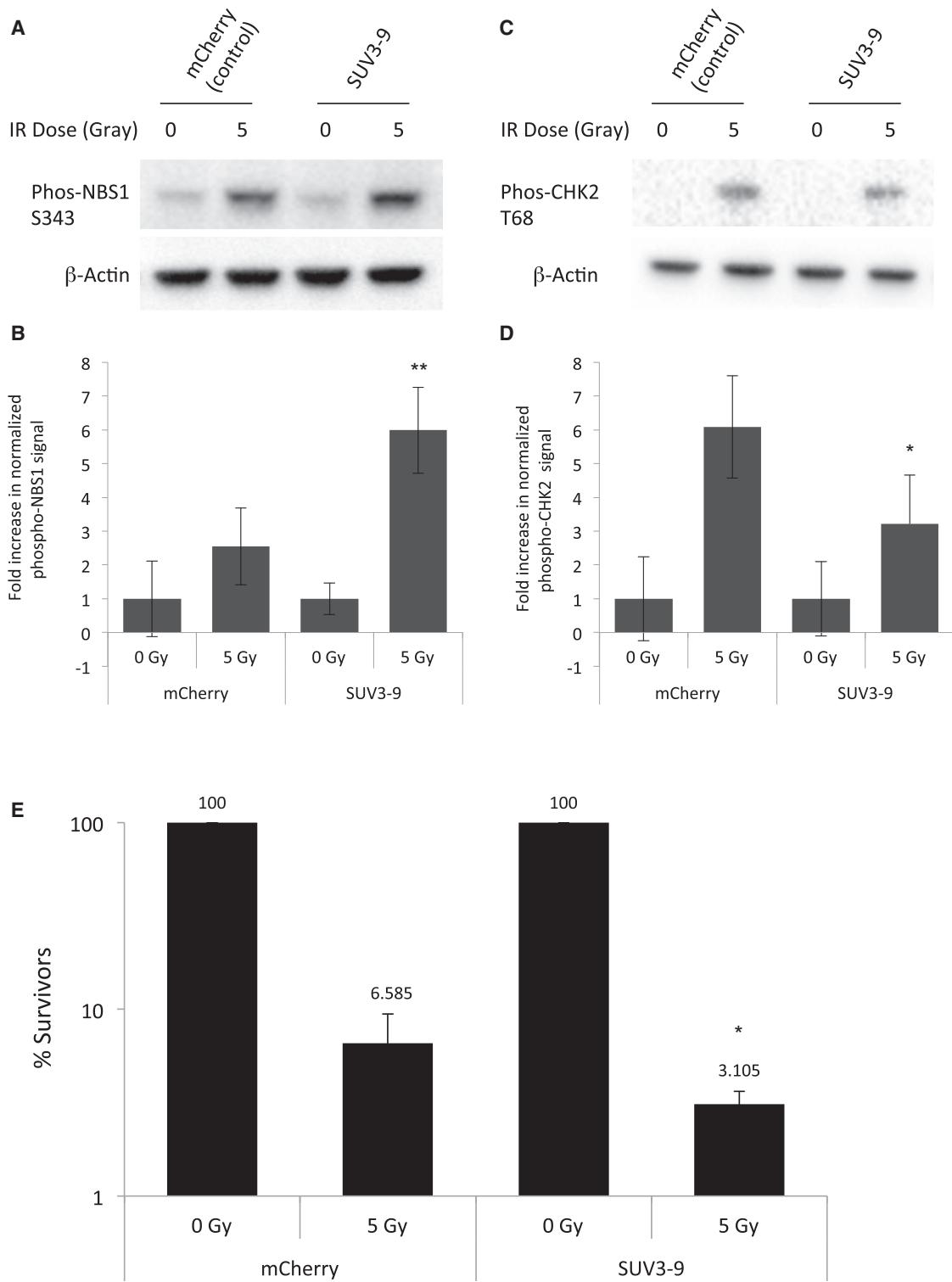


Figure 6. Persistent Condensation of Chromatin Enhances Upstream Signaling from Ionizing-Radiation-Induced Breaks but Reduces Cell Survival after Damage

(A) Immunoblot of phospho-NBS1 in control or *SUV3-9*-overexpressing cells \pm 5 Gy γ irradiation. β -actin shown as a loading control.

(B) Quantification of phospho-NBS1 levels, normalized to β -actin. Values represent averages \pm SD from three experiments. Unirradiated NBS1 levels are normalized to 1. ** p < 0.01 compared to irradiated control.

(C) Western blot of phospho-CHK2 in conditions as in (A).

(legend continued on next page)

exclusive, the temporal coordination of chromatin dynamics may be complemented by spatial separation of decondensed and condensed chromatin domains in the vicinity of a DSB, with relaxed and compact regions playing distinct roles in DDR signaling and repair. In fact, subcompartments within DNA damage foci have been observed by superresolution microscopy and their chromatin environments proposed to be distinct (Chapman et al., 2012). Establishment of repressive chromatin may be beneficial to the damaged region to stabilize the damaged ends and concentrate DDR factors for more-efficient signaling. In addition, condensed domains may contribute to the transcriptional repression characteristic of damaged genome regions and help keep the transcription machinery from interfering with downstream repair processes (Kruhlak et al., 2007; Pankotai et al., 2012; Price and D'Andrea, 2013; Shanbhag et al., 2010).

Taken together, these observations provide evidence that chromatin condensation is an integral but transient step in the activation of DDR signaling, integrating observations of opposing dynamics of chromatin after damage. Our data allow for the possibility that, in addition to detecting bona fide DNA damage, the cellular surveillance machinery also senses changes in chromatin structure.

EXPERIMENTAL PROCEDURES

Laser Microirradiation

Parental U2OS cells were transfected according to manufacturer's protocol with Amaxa nucleofector V (Lonza) with a photoactivatable GFP-tagged H2A with or without DNA repair factors or chromatin modifiers and plated on two-chamber coverslip bottom slides (Lab-tek) for 20 hr. Before imaging, cells were incubated with 0.1 µg/ml Hoechst 33342 for 1 hr and then switched to FluoroBrite phenol red-free media containing 10% fetal bovine serum, glutamine, and antibiotics without Hoechst and 5 mM HEPES. Imaging and laser damage/photoactivation was carried out as described previously on a Zeiss LSM510 with a 364 nm laser (Kruhlak et al., 2006) or on a Zeiss LSM780 with simultaneous 355 nm (10%) and 405 nm (5%) laser lines, with total UV laser output set to 20%, ten iterations, and laser scan speed set to 7 (pixel dwell time 3.15 µs). The laser(s) were focused in a 30-pixel circle and images taken every 10 s for 2 min then every 45 s for 30 min, maintaining cells at 37°C and 5% CO₂. Subsequent region area measurements were performed using ImageJ software.

Ligation-Mediated PCR

Genomic DNA was purified from U2OS cell lines using the QIAGEN Blood and Cell Culture DNA Mini Kit and prepared for ligation-mediated PCR to detect random array breaks or I-SceI-induced breaks as described previously (Soutoglou et al., 2007; Soutoglou and Misteli, 2008), respectively.

Chromatin Factor Tethering

Transient transfections were carried out using the Amaxa Nucleofector Kit V (Lonza), according to the manufacturer's protocol. All tethering experiments were carried out using transient expression of the constructs for 20 hr before harvesting or fixation, unless otherwise noted. For I-SceI expression, cells were transfected first with tethering constructs using the Amaxa Nucleofector kit V and maintained in charcoal-stripped serum (Atlanta Biologicals). Twelve hours later, cells were transfected with cyan fluorescent protein (CFP)-GR-I-SceI construct per 1 million cells using the same protocol. After 12 hr (24 hr

total), GR nuclear translocation was induced with dexamethasone (Sigma-Aldrich) at a concentration of 100 nM for 20 min.

Immunofluorescence and Imaging

Cells were fixed and stained as previously described (Soutoglou et al., 2007) except that images were captured on a DeltaVision workstation equipped with a CCD camera (CoolSNAP HQ, Photometrics) mounted on a microscope (IX70, Olympus) with a 60 × 1.42 NA oil immersion objective (Olympus). Twenty to fifty focal planes were captured at 0.2–0.5 µm resolution and analyzed with the softWoRx package for colocalization or total nuclear H2AX (Applied Precision) or ImageJ (NIH) for integrated density measurements of foci.

Kinase Inhibitor Treatment

Kinase inhibitors were added to medium just after nucleofector transfection with the tethering constructs (see above), at the following final concentrations: KU55933, 10 µM; VE-821, 1 µM; and NU7441, 1 µM. Cells were fixed and stained after 20 hr of incubation, as described above.

siRNA Knockdown

Dharmafect On-Target-plus SMART-pool siRNAs for ATM and ATR were used. One hundred nanomolar siRNAs were cotransfected with tethering constructs with Dharmafect1, according to the manufacturer's instructions, and harvested for protein analysis and microscopy after 72 hr.

Clonogenic Survival Assays

Assay was carried out essentially as described previously (Munshi et al., 2005), with the following changes: four million cells were transfected with overexpression constructs, plated in T75 flasks, and incubated for 20 hr posttransfection for full expression. Cells were irradiated with 5 Gy of γ irradiation using a ¹³⁷Cs source and then trypsinized, cells counted, and plated at two cell densities, each in triplicate on 10 cm plates in standard medium. After 12–14 days of growth, colonies were fixed and stained with 0.25% crystal violet in ethanol before counting.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2014.10.060>.

AUTHOR CONTRIBUTIONS

R.C.B and T.M. designed the study; R.C.B., B.B., and M.J.K. performed experiments and analyzed data; and R.C.B. and T.M. wrote the manuscript.

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(D) Quantification of phospho-CHK2 as in (B). *p < 0.05 compared to irradiated control.

(E) Clonogenic survival assays of cells expressing SUV3-9. Surviving colonies were normalized to unirradiated controls. Values represent median ± median average deviation from three experiments. *p < 0.05 compared to irradiated control.

See also Figure S6.

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