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The Chromatin Scaffold Protein SAFB1 Renders Chromatin Permissive for DNA Damage Signaling

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SUMMARY

Although the general relevance of chromatin modifications for genotoxic stress signaling, cell-cycle checkpoint activation, and DNA repair is well established, how these modifications reach initial thresholds in order to trigger robust responses remains largely unexplored. Here, we identify the chromatin-associated scaffold attachment factor SAFB1 as a component of the DNA damage response and show that SAFB1 cooperates with histone acetylation to allow for efficient γH2AX spreading and genotoxic stress signaling. SAFB1 undergoes a highly dynamic exchange at damaged chromatin in a poly(ADP-ribose)-polymerase 1- and poly(ADP-ribose)-dependent manner and is required for unperturbed cell-cycle checkpoint activation and guarding cells against replicative stress. Altogether, our data reveal that transient recruitment of an architectural chromatin component is required in order to overcome physiological barriers by making chromatin permissive for DNA damage signaling, whereas the ensuing exclusion of SAFB1 may help prevent excessive signaling.

INTRODUCTION

DNA damage occurs from various exogenous and endogenous sources and, if left unrepaired or incorrectly mended, can cause genomic instability and result in the development of human diseases, including immunodeficiency, neurodegeneration, premature aging, and cancer (Jackson and Bartek, 2009). To counteract genotoxic stress and prevent chromosome fragility, cells have developed sophisticated genome surveillance and repair mechanisms (Ciccia and Elledge, 2010). DNA-damage-induced chromatin modifications have emerged as important regulatory mechanism for shielding broken chromosomes from degradation, efficiently recruiting DNA repair factors to the damaged regions, and coordinating repair events with other ongoing chromatin transactions (Lukas et al., 2011; Polo and Jackson, 2011). All major types of posttranslational modifications are involved in the chromatin response to DNA breakage, including chromatin phosphorylation, ubiquitylation, sumoylation, and poly(ADP ribosyl)ation (PARylation).

Chromatin modifications after the infliction of DNA damage must be robust and occur quickly in order to prevent illegitimate chromosome fusions or detrimental nucleolytic attrition of chromosome ends. However, physiological barriers exist that most likely evolved to guard against the excessive spreading of chromatin modifications (Altmeyer and Lukas, 2013; Gudjonsson et al., 2012). How these natural constraints are overcome to amplify the signal to the required threshold and to what degree they regulate the extent of DNA damage signaling has remained largely unknown.

The scaffold attachment factor B1 (SAFB1) is a nonenzymatic architectural component of the chromatin that was previously shown to bind adenine- and thymine-rich scaffold/matrix attachment (S/MAR) regions (Renz and Fackelmayer, 1996), which are believed to partition the genome into 5–200 kb topological domains, and SAFB1 was proposed to mediate chromatin looping in order to regulate long-range chromatin interactions and higher-order chromatin structure (Garee and Oesterreich, 2010). SAFB1 coregulates gene expression and couples RNA-polymerase II-dependent transcription to splicing in transcriptosomal complexes (reviewed in Garee and Oesterreich, 2010). In particular, SAFB1 can bind to nuclear receptors (Debril et al., 2005; Oesterreich et al., 2000) and corepress immune regulators and apoptotic genes (Hammerich-Hille et al., 2010b). On the basis of frequent loss of heterozygosity and low expression in breast cancer tissues, SAFB1 was proposed as a candidate tumor suppressor gene (Oesterreich, 2003; Oesterreich et al., 2001). Later, low levels of SAFB1 were found to correlate with worse outcomes in breast cancer patients (Hammerich-Hille et al., 2010a). *Safb1*$^{-/-}$ mice show pre- and neo-natal lethality, growth retardation, male infertility, female subfertility, and

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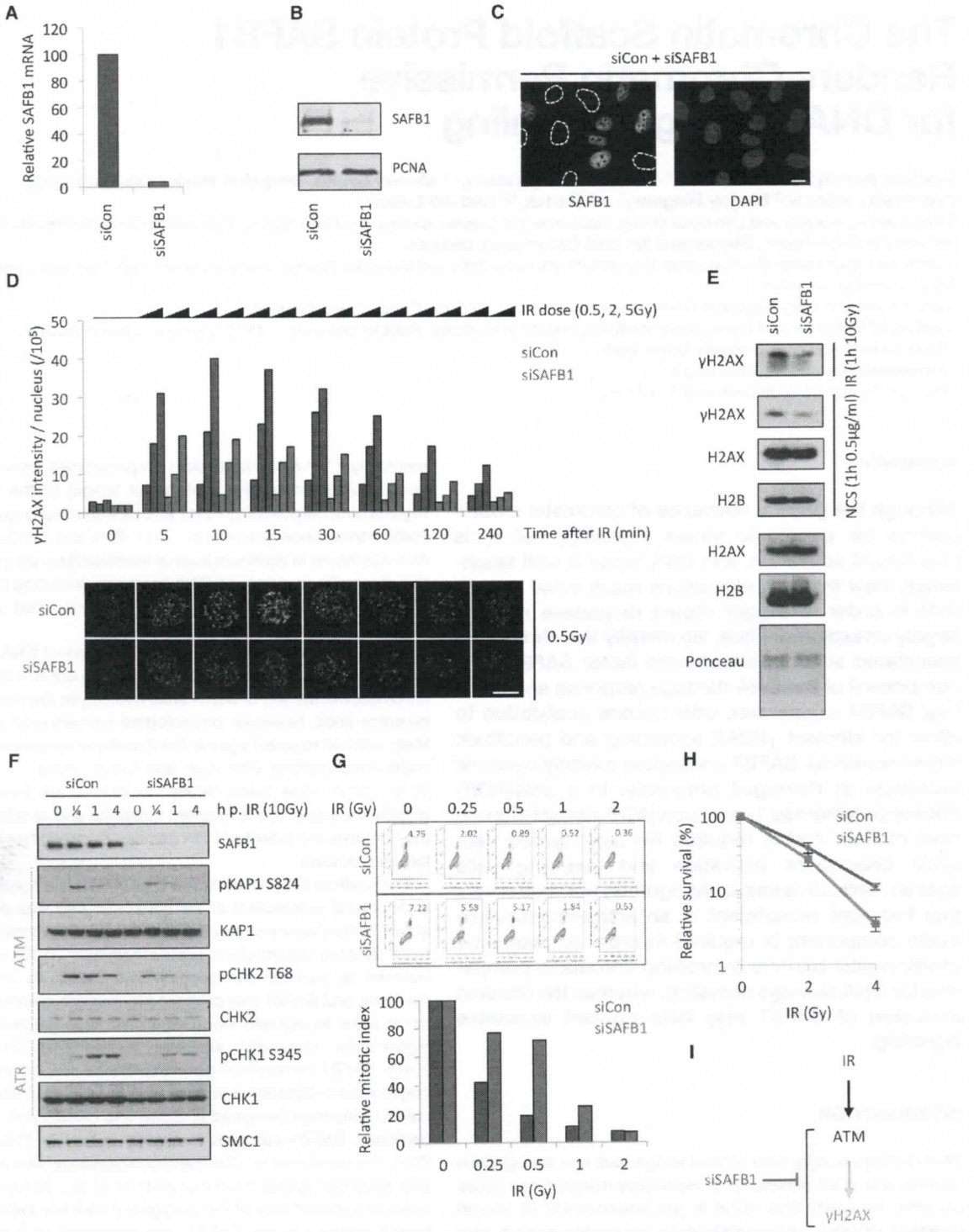


Figure 1. SAFB1 Facilitates γH2AX Formation after DNA Breakage

(A–C) SAFB1 knockdown efficiency 72 hr after transfection analyzed by quantitative RT-PCR (A), western blot (B), and immunofluorescence staining of mixed control siRNA and SAFB1 siRNA-treated cells (C).

(D) Cells were transfected with siRNA and treated as indicated; irradiated with 0.5, 2, or 5 Gy; fixed 0–240 min post IR; and stained for γH2AX. Nuclear γH2AX intensities were quantified by automated high-content image analysis.

(legend continued on next page)

immune defects and are prone to tumor development (Ivanova et al., 2005). More recently, SAFB1 appeared in several unbiased genotoxic-stress-related RNAi and mass-spectrometry-based screens (Beli et al., 2012; Bennetzen et al., 2010; Bensimon et al., 2010; Chou et al., 2010; Gagné et al., 2012; Paulsen et al., 2009). These links, along with the proposed function of SAFB1 in mediating chromatin looping and higher-order chromatin structure, prompted us to address whether SAFB1 would be involved in the chromatin response to DNA damage.

RESULTS

SAFB1 Is Required for Efficient H2AX Phosphorylation in Response to DNA Double-Strand Breaks

To test whether SAFB1 would play a role in the regulation of DNA-damage-induced chromatin modifications, we depleted SAFB1 from U-2-OS cells by small interfering RNA (siRNA). SAFB1 messenger RNA (mRNA) and protein levels were greatly reduced, as revealed by quantitative RT-PCR (Figure 1A), western blot (Figure 1B), and immunofluorescence (IF) staining of mixed control siRNA and SAFB1 siRNA-treated cells 3 days after transfection (Figure 1C). Next, we treated cells with ionizing radiation (IR) to induce DNA double-strand breaks (DSBs) and performed IF staining for γH2AX, the most apical chromatin modification generated by the ATM kinase in response to DNA damage. Interestingly, whereas ATM inhibition largely abolished γH2AX formation under these conditions, SAFB1 depletion also reduced H2AX phosphorylation, indicating that SAFB1 is required for efficient γH2AX formation after IR (Figure S1A available online). Then, we employed automated microscopy combined with software-assisted image analysis to obtain sensitive and quantitative measurements of SAFB1-dependent γH2AX formation in large cohorts of cells (see the Supplemental Experimental Procedures). An extensive analysis with three different IR doses ranging from 0.5–5 Gy and eight different time points from 0–240 min after IR revealed that, under all conditions, SAFB1-depleted cells had reduced amounts of IR-induced γH2AX (Figure 1D). Remarkably, even at the level of γH2AX intensity per individual subnuclear focus, SAFB1-depleted cells showed reduced spreading of chromatin phosphorylation (Figure S1B), resulting in lower counts of cytologically discernible foci (Figure S1C). Importantly, these effects were independent of cell-cycle stage (Figure S1D), could be recapitulated by a second independent siRNA against SAFB1 (Figure S1E), and were rescued by the re-expression of siRNA-resistant SAFB1 (Figure S1F). Furthermore, H2AX isolated from chromatin by acid extraction was less phosphorylated in response to DSB induction after SAFB1 depletion, whereas the total amount of chromatinized H2AX was comparable between SAFB1-depleted and control siRNA-treated cells (Figure 1E). Altogether, these experiments suggest that the nonhistone chromatin component SAFB1 is required for efficient γH2AX formation and spreading in response to clastogen-induced DSBs.

H2AX phosphorylation is essential for the self-reinforced amplification of DNA damage response (DDR) signaling. Consequently, the loss of H2AX causes DNA damage checkpoint defects, especially in response to low doses of IR (Fernandez-Capetillo et al., 2002), and H2AX haploinsufficiency compromises genome integrity and can lead to increased tumor susceptibility (Celeste et al., 2003). Therefore, we tested whether the reduced γH2AX levels in SAFB1-depleted cells would also impact on DNA damage signaling and checkpoint activation. Indeed, the immediate IR-induced phosphorylation of the ATM targets KAP1 and CHK2 as well as the delayed phosphorylation of the ATR target CHK1 were reduced in SAFB1-depleted cells (Figure 1F). Moreover, consistent with impaired chromatin phosphorylation in response to DNA breakage, SAFB1-depleted cells were less efficient in triggering the G2/M checkpoint after low doses of IR (Figure 1G) and showed slightly reduced colony formation after IR in long-term clonogenic survival assays (Figure 1H) but had no signs of apoptosis in the absence of exogenous genotoxic stress (Figure S1G). Altogether, these results suggest that SAFB1 contributes to the magnitude of γH2AX formation and is required for proper DNA damage signaling, checkpoint activation, and cell survival in response to ionizing radiation (Figure 1I).

SAFB1 Is Required for Replication Fork Stability

SAFB1 depletion also resulted in reduced levels of γH2AX when cells were treated with the DNA-damaging drug neocarzinostatin (Figures 1E and S1H) or with the topoisomerase II inhibitor etoposide, a potent inducer of DSBs at regions of topological problems arising during transcription and DNA replication (Figure S1I). These results (especially the latter) suggested that the requirement for SAFB1 to trigger an efficient DDR was not limited to clastogen-induced DSBs but might be a more general feature of the chromatin response to stress assaults; for instance, those associated with DNA replication.

To test this hypothesis, we took advantage of previous findings describing the temporal development of replication stress and its impact on H2AX phosphorylation (Figure 2A). Specifically, upon replication fork stalling, cells elicit a rapid ATR-dependent response resulting in feed-forward γH2AX formation and CHK1 activation (Kerzendorfer and O'Driscoll, 2009). If replication stalling persists or if ATR signaling becomes inefficient, then replication forks eventually collapse into DSBs, leading to ATM activation and, consequently, greatly increased γH2AX production. Indeed, when we applied high-content microscopy to specifically evaluate signaling in S phase cells exposed to replication fork stalling induced by hydroxyurea (HU), we observed that the

(E) Cells were transfected and treated with IR or neocarzinostatin (NCS) as indicated. Histones were isolated by acid extraction and analyzed by western blot.
(F) Cells were treated as indicated, and whole-cell extracts were analyzed by western blot. ATM- and ATR-mediated phosphorylations are indicated.
(G) Cells were irradiated as indicated, and, 4 hr later, nocodazole-blocked cells were harvested for flow cytometry analysis of histone H3 Ser10 phosphorylation as a marker of G2/M checkpoint inefficiency.
(H) Clonogenic survival of siControl and siSAFB1 transfected cells after IR. Data represent means ± SD.
(I) Schematic representation of SAFB1 function for γH2AX formation after IR. Scale bars represent 10 μm.
See also Figure S1.

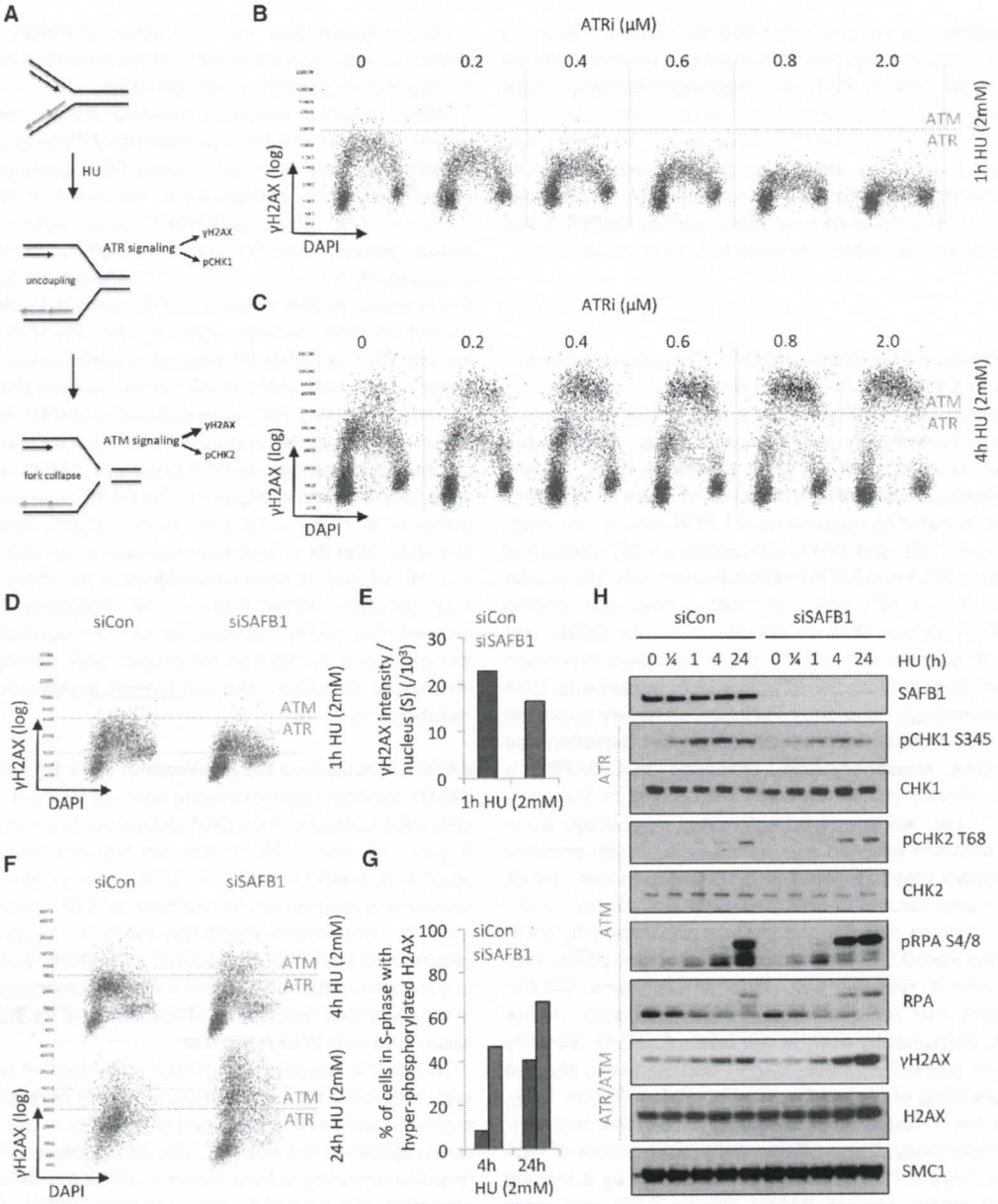


Figure 2. SAFB1 Promotes Replication Fork Stability

(A) Model illustrating major kinase signaling events at stalled and broken replication forks.

(B) Cells were treated for 1 hr with HU in the presence of increasing concentrations of ATR inhibitor. Nuclear γH2AX intensities were analyzed by automated high-content imaging. ATM- and ATR-mediated H2AX phosphorylation is indicated.

(C) Cells were treated for 4 hr with HU in the presence of increasing concentrations of ATR inhibitor. Nuclear γH2AX intensities were analyzed by automated high-content imaging. ATM- and ATR-mediated H2AX phosphorylation is indicated.

(D) Cells were transfected with siRNA as indicated and treated for 1 hr with HU, and nuclear γH2AX intensities were analyzed by automated high-content imaging. ATM- and ATR-mediated H2AX phosphorylation is indicated.

(E) Quantification of γH2AX levels in S phase cells.

(F) Cells were transfected as indicated and treated for 4 hr with HU, and nuclear γH2AX intensities were analyzed by automated high-content imaging. ATM- and ATR-mediated H2AX phosphorylation is indicated.

(G) Quantification of S phase cells with hyperphosphorylated H2AX.

(H) Cells were treated with 2 mM HU as indicated, and whole-cell extracts were analyzed by western blot. ATM- and ATR-mediated phosphorylations are indicated.

See also Figure S2.

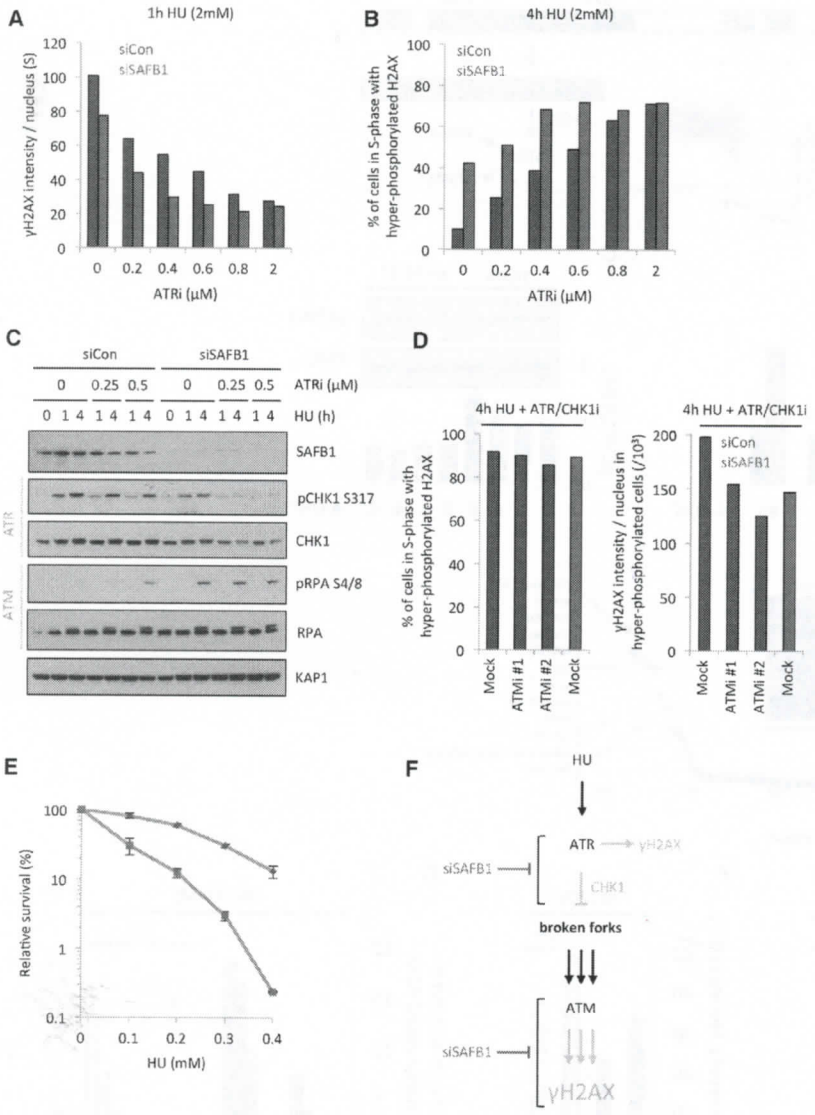


Figure 3. SAFB1 Contributes to the Magnitude of ATR Signaling from Stalled Replication Forks and to ATM Signaling from Broken Forks

(A) Cells were transfected with siRNA as indicated and treated for 1 hr with HU in the presence of increasing concentrations of ATR inhibitor, and γH2AX levels in S phase cells were quantified.

(B) Cells were transfected as indicated and treated for 4 hr with HU in the presence of increasing concentrations of ATR inhibitor, and S phase cells with hyperphosphorylated H2AX were quantified.

(C) Cells were transfected and treated as indicated with 2 mM HU and different concentrations of ATR inhibitor, and whole-cell extracts were analyzed by western blot. ATM- and ATR-mediated phosphorylations are indicated.

(D) Cells were transfected as indicated and treated for 4 hr with 2 mM HU in the presence of an ATR and CHK1 inhibitor cocktail to effectively force cells into replication-fork-collapse-induced DNA breakage. S phase cells with hyperphosphorylated H2AX (left), and γH2AX levels in hyperphosphorylated cells (right) were quantified; 2 μM ATR inhibitor and 300 nM CHK1 inhibitor UCN-01 were used.

(E) Clonogenic survival of HU-treated siControl- and siSAFB1-transfected cells. Data represent means ± SD.

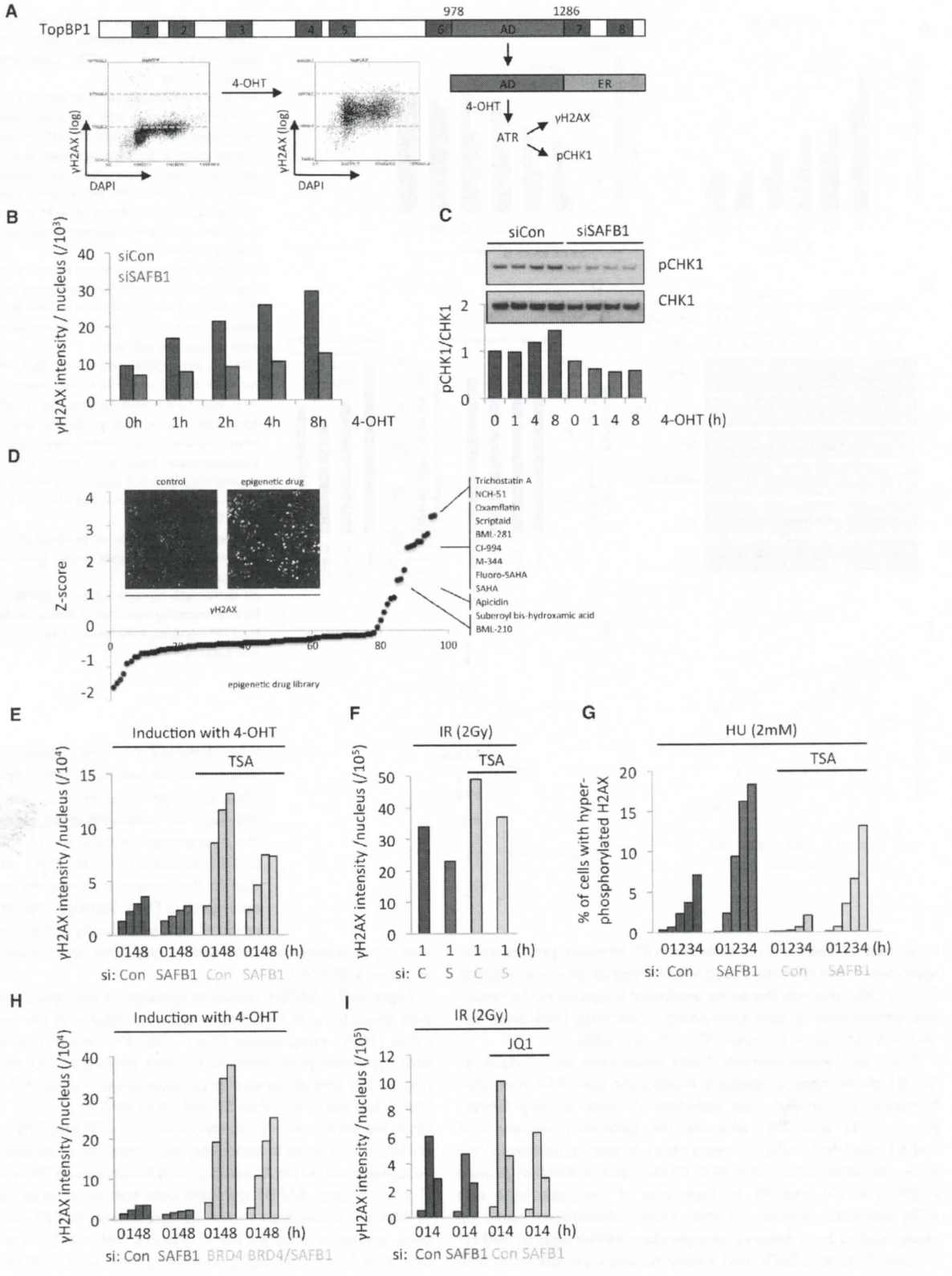
(F) Schematic representation of SAFB1 function for ATR signaling from stalled replication forks and for ATM signaling from broken forks.

See also Figure S3.

treatment of cells with a specific ATR inhibitor progressively suppressed the ATR-mediated γH2AX formation at early stages (Figure 2B), whereas the same treatment triggered H2AX hyperphosphorylation at later time-points, indicating DNA breakage and ATM activation (Figures 2C, S2A, and S2B).

Then, we tested whether these responses would have an SAFB1-dependent component. Indeed, the early ATR-mediated formation of γH2AX was reduced in cells lacking SAFB1 (Figures 2D and 2E), whereas the extended exposure of SAFB1-depleted cells to replication stress accelerated the hyperphosphorylation of H2AX, CHK2, and replication protein A (RPA) at S4 and S8, all hallmarks of DNA breakage and ATM activation (Figures 2F–2H). These phenotypes could be recapitulated by a second, independent siRNA against SAFB1 (Figures S2C and S2D), and similar results were obtained with the DNA polymerase inhibitor aphidicolin (Figure S2E) and the topoisomerase I inhibitor camptothecin, which also causes replication-mediated DNA damage (Figures S2F and S2G). Although we noticed a slight increase in the percentage of cells in G1 following SAFB1 depletion (Figure S3A), possibly due to the progressive accumulation of endogenous DNA damage, the incorporation of EdU specifically in S phase cells was comparable between SAFB1-depleted and control cells (Figures S3B–S3D).

Importantly, SAFB1 depletion synergized with low doses and was epistatic with high doses of ATR inhibitor at the levels of initial γH2AX suppression (Figure 3A), the percentage of cells with hyperphosphorylated H2AX after prolonged HU exposure (Figure 3B), and at the level of phosphorylation of the ATR target CHK1 and the ATM target S4 and S8 of RPA (Figure 3C). Finally, when we combined HU treatment with an ATR and CHK1 inhibitor cocktail in order to effectively force cells into replication-fork-collapse-induced DNA breakage and the ensuing ATM activation (Figure 3D, left), SAFB1-depleted cells had reduced amounts of γH2AX in comparison to the corresponding SAFB1-proficient cells, similar to what we could observe with two different ATM inhibitors (Figure 3D, right), indicating that, even after enforced premature replication fork breakage, SAFB1 deficiency restrains



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γH2AX spreading. Finally, consistent with a protective role of SAFB1 against replication stress, SAFB1-depleted cells showed reduced clonogenic survival in response to HU (Figure 3E). Altogether, our results suggest that the role for SAFB1 in facilitating γH2AX formation extends beyond clastogen-induced DSBs and contributes to the magnitude of ATR signaling from stalled replication forks as well as to ATM signaling from broken forks (Figure 3F).

The SAFB1-Mediated Effect on γH2AX Formation Cooperates with Chromatin Acetylation

Given that the expression levels of the major DNA-damage-induced protein kinases ATM, ATR, DNA-PK, and their downstream effectors CHK1 and CHK2 seemed comparable in SAFB1-deficient and SAFB1-proficient cells (Figures S4A and S4B) and that whole-genome expression arrays did not indicate any role for SAFB1 in regulating the expression of DNA repair genes (Hammerich-Hille et al., 2010b), the above findings suggested that SAFB1 might influence chromatin topology in a manner favorable for efficient genotoxic stress signaling and γH2AX formation. To investigate this issue further, we turned to a system that allows for the regulated activation of DDR signaling independent of DNA break formation and independent of DNA replication (Toledo et al., 2008). In this cellular model system, the DDR kinase ATR is activated by the inducible nuclear import of the TopBP1 ATR activation domain (AD). Indeed, when we used this system and analyzed γH2AX levels prior to and after induction by automated imaging, we observed a cell-cycle-stage-independent increase in γH2AX production (Figure 4A). Strikingly, under these conditions, SAFB1 was required to elicit an efficient time-dependent response (Figures 4B and 4C). These data strongly reinforce the notion that reduced γH2AX formation in SAFB1-depleted cells is not due to reduced break formation or differences in cell-cycle progression but, rather, reflects a chromatin conformation that is less permissive for the sustained activation of DDR kinases and spreading of the γH2AX signal. First, to extend and further investigate this regulation, we performed a targeted epigenetic and protease inhibitor drug screen in SAFB1-proficient cells with the aim of identifying chemical compounds that would modulate the TopBP1 ATR activation-construct-induced, DNA-damage- and replication-independent γH2AX response. This drug library screen revealed that the twelve top scoring compounds with the ability to enhance γH2AX formation under these conditions were all inhibitors of histone deacetylases (HDACs) (Figures 4D and Table S1).

Next, intrigued by this result, we tested whether histone acetylation and SAFB1 function might cooperate to render chromatin permissive for γH2AX formation. We treated U-2-OS cells for 5 hr with a low concentration (100 nM) of the HDAC inhibitor trichostatin A (TSA), a treatment that enhanced global histone H4K16 acetylation levels (Figure S4C) but did not cause measureable cell-cycle checkpoint activation (Figure S4D). Although TSA treatment enhanced γH2AX formation in the TopBP1 AD system, thus confirming the result from the initial drug screen and indicating that ATR target phosphorylation responds to changes in chromatin structure, SAFB1 depletion counteracted this effect (Figure 4E). Similar results were observed after ionizing radiation (Figure 4F) and upon HU exposure (Figure 4G). Also, when treated with the genotoxic agent neocarzinostatin, exposure to TSA or SAHA, a second HDAC inhibitor that scored in our drug screen, resulted in enhanced γH2AX formation in a dose-dependent manner, and SAFB1 depletion mitigated this effect (Figures S4E and S4F). These results are consistent with previous findings showing that chemically enforced histone hyperacetylation and chromatin decompaction increase γH2AX formation (Murga et al., 2007) and suggest that the SAFB1-mediated enhancement of γH2AX signaling cooperates with histone acetylation to overcome chromatin constraints that limit DDR signaling.

One particular chromatin component that shields against γH2AX spreading and DDR signaling was recently identified in the bromodomain protein BRD4 (Floyd et al., 2013). Indeed, when we depleted BRD4 from U-2-OS cells and analyzed DNA-damage- and replication-independent γH2AX formation in the TopBP1 AD system, we observed a marked increase in H2AX phosphorylation, an effect that could be attenuated by the codepletion of SAFB1 (Figure 4H). Similarly, the exposure of cells to JQ1, a small-molecule inhibitor of BET bromodomains (Filippakopoulos et al., 2010), enhanced γH2AX formation after IR, and this effect was also dampened in SAFB1-depleted cells (Figure 4I). Altogether, these results suggest that SAFB1 antagonizes chromatin constraints such as hypoacetylation and chromatin insulation in order to assist the DDR in overcoming structural barriers to trigger a robust and efficient chromatin response.

SAFB1 Undergoes a Dynamic Exchange at Damaged Chromatin, Including Transient Recruitment Followed by Sustained Exclusion

To elucidate whether SAFB1 exerts its role for efficient γH2AX formation globally throughout the nucleus or directly at the damaged chromatin, we analyzed SAFB1 localization in

Figure 4. The SAFB1-Mediated Effect on γH2AX Formation Cooperates with Chromatin Acetylation

(A) The TopBP1 ATR activation domain (AD) was used to induce cell-cycle-phase- and DNA-damage-independent γH2AX formation.

(B) Cells stably expressing the TopBP1 ATR activation construct were transfected as indicated, induced for 0–8 hr with 4-OHT, and stained for γH2AX.

(C) Cells stably expressing the TopBP1 ATR activation construct were treated as indicated, whole-cell extracts were analyzed by western blot, and relative pCHK1 levels were quantified.

(D) Cells stably expressing the TopBP1 ATR activation construct were induced for 8 hr in the presence of a small drug library and stained for γH2AX.

(E) Cells stably expressing the TopBP1 ATR activation construct were treated as indicated with 4-OHT with or without 100 nM TSA for 5 hr and stained for γH2AX.

(F) Cells were transfected with siRNA as indicated, treated with 2 Gy, fixed 1 hr after IR, and stained for γH2AX. C, Con; S, SAFB1.

(G) Cells were transfected as indicated and treated with HU, and cells with ATM-mediated hyperphosphorylation of H2AX were quantified.

(H) Cells stably expressing the TopBP1 ATR activation construct were transfected and treated as indicated and stained for γH2AX.

(I) Cells were transfected and treated as indicated with or without 1 μM JQ1 for 8 hr, exposed to 2Gy as indicated, and stained for γH2AX.

See also Figure S4 and Table S1.

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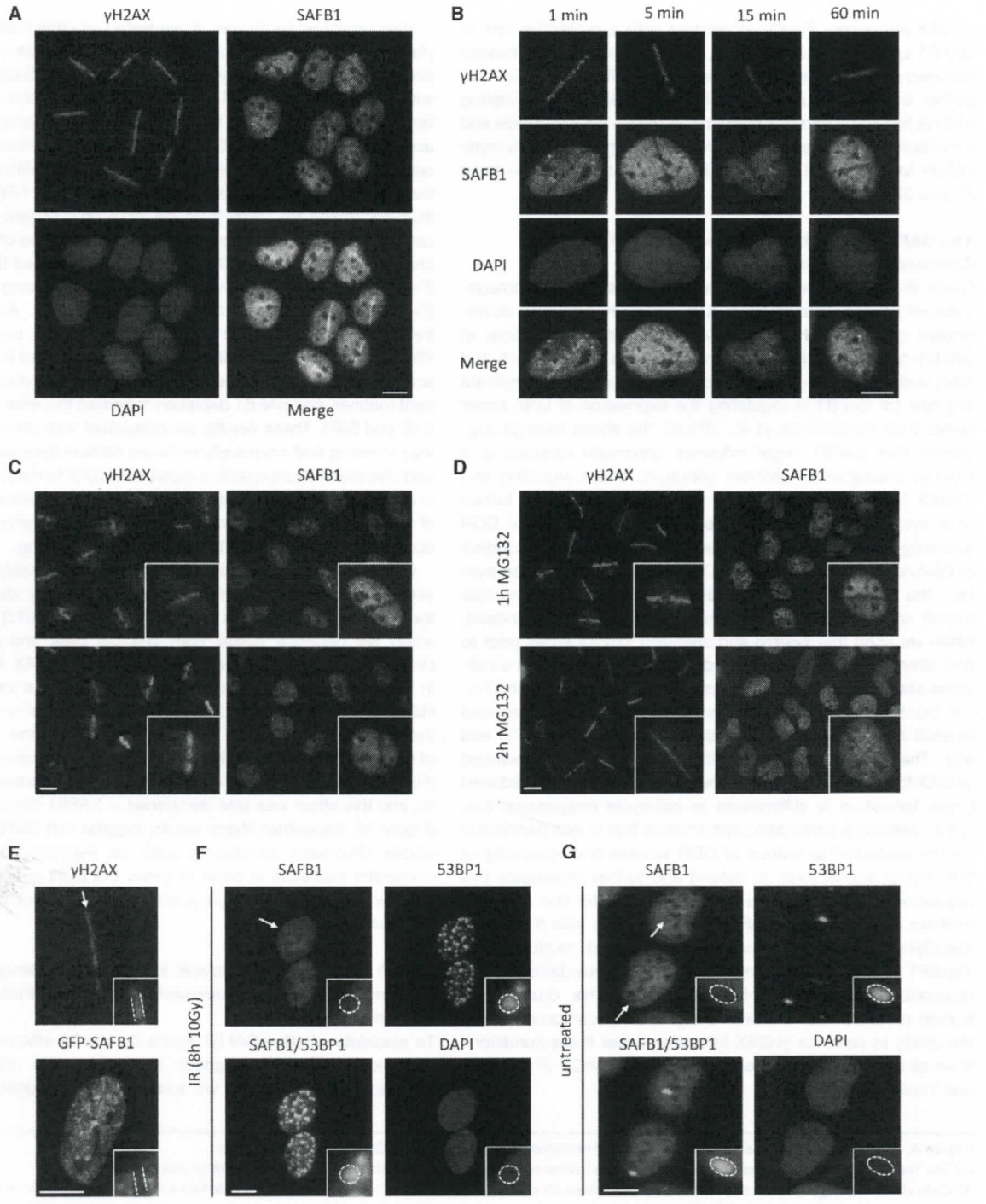

Figure 5. SAFB1 Is Excluded from Damaged Chromatin

(A) Localized DNA damage was induced by laser microirradiation. Cells were fixed and stained for γH2AX and SAFB1 15 min after damage induction.

(B) Time course of SAFB1 antistripe formation. Representative cells are shown.

(C) The same experiments were performed as in (A) and (B) with swapped fluorophores to detect γH2AX and SAFB1, respectively.

(D) Cells were pretreated with 10 μM of the proteasome inhibitor MG132 prior to laser damage and stained for γH2AX and SAFB1.

(E) GFP-SAFB1-expressing cells were laser microirradiated and stained for γH2AX.

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response to DNA damage by laser microirradiation. We employed laser settings that did not cause excessive spreading of DDR factors beyond their physiological boundaries or result in excessive DNA-end resection, as indicated by the locally confined and S/G2-phase-specific accumulation of the single-stranded DNA binding protein RPA (Figures S5A–S5D). Unexpectedly, we found that, under these conditions, SAFB1 showed negative staining around DNA damage sites (Figure 5A). SAFB1 disappearance from damaged areas was a fast response observed minutes after damage induction (Figure 5B) and was neither due to staining or FRET artifacts, as revealed by dye-swap experiments (Figure 5C), nor local protein degradation, given that the pretreatment of cells with the proteasome inhibitor MG132 did not reduce SAFB1 antistripe formation (Figure 5D). Finally, ectopically expressed GFP-SAFB1 also showed antistripes at damaged areas, indicating bona fide SAFB1 displacement from DNA lesions (Figure 5E and Movie S1). Importantly, SAFB1 removal was not only observed around sites of laser microirradiation but also at damaged chromatin regions after IR (Figure 5F) and at sites of spontaneous, replication-induced damage, which manifest in G1 cells after the completion of mitosis (Figure 5G). Altogether, these results suggest that SAFB1 is readily mobilized in response to DNA damage and becomes largely excluded from DNA damage sites.

The above findings raised the question of how an architectural chromatin component could assist γH2AX formation if the protein itself was not present at the site of action. Intrigued by this conundrum, we noticed that two recent reports had identified components of the DDR network that showed remarkable dual recruitment and exclusion behavior in response to DNA damage (Adamson et al., 2012; Polo et al., 2012). Inspired by these findings, we tested whether a similar redistribution behavior could also be attributed to SAFB1. Indeed, when we combined mild detergent-mediated pre-extraction with RNase digestion as performed by Polo et al. (2012) to remove the soluble and RNA-bound protein pool, we observed that the remaining SAFB1 was transiently recruited to sites of laser microirradiation followed by persistent release from the damaged area (Figure 6A). This suggests that a fraction of SAFB1, most likely containing SAFB1 molecules not involved in RNA-dependent processes such as transcription or splicing, is not expelled from damaged chromatin but can transiently accumulate around DNA lesions. We noticed considerable cell-to-cell variation in the accumulation of SAFB1 at damaged chromatin, which probably reflects the high degree of heterogeneity in gene expression and splicing patterns that can be seen between cells (Shalek et al., 2013).

Importantly, neither the inhibition of the DDR kinases ATM, ATR, and DNA-PK nor the depletion of the downstream ubiquitin ligases RNF8 and RNF168 could abolish SAFB1 recruitment (data not shown and see below). Conversely, the addition of Mirin, a previously identified inhibitor of MRE11 (Dupré et al., 2008), just minutes before laser microirradiation enhanced the early SAFB1 accumulation at damaged sites in a dose-dependent manner and at already relatively low concentrations (Figure 6B). Although the cellular activities of Mirin could potentially extend beyond MRE11 function and might, for instance, also affect other DNA endonucleases, these results suggest that blocking DNA repair processes can robustly expose SAFB1 accumulation and that such treatment could provide a useful tool for dissecting the upstream events that regulate SAFB1 recruitment.

Given that chromatin phosphorylation by ATM, ATR, and DNA-PK and ubiquitylation by RNF8 and RNF168 did not seem to be required for SAFB1 recruitment, we focused on a parallel pathway that is rapidly activated in response to DNA breakage and regulates the fast recruitment of various chromatin modifiers, remodelers, and repair factors to damage sites, namely PARylation (Altmeyer and Lukas, 2013). We noticed that Mirin treatment not only stabilized MRE11 at sites of laser microirradiation (data not shown) but also enhanced the binding of poly(ADP-ribose) (PAR) polymerase 1 (PARP1, also referred to as ARTD1) (Hottiger et al., 2010) to DNA lesions (Figure S6A) and even increased damage-induced PARylation (Figure S6B). Stimulated by this result, we tested whether PARylation might be involved in regulating SAFB1 recruitment. Again, we used pre-extraction and RNase digestion combined with Mirin treatment in order to achieve robust SAFB1 recruitment to damage sites and then applied PARP inhibitors or siRNA against PARP enzymes to test their impact on SAFB1 relocalization. Indeed, the two different PARP inhibitors tested, and PARP1 and PARP2 codepletion by siRNA completely abolished SAFB1 recruitment under these conditions (Figure 6C). Conversely, knockdown of PAR glycohydrolase (PARG), the major antagonist of PARP-dependent chromatin PARylation, enhanced SAFB1 recruitment to such an extent that association with damaged areas could be readily observed even without pre-extraction and RNase digestion (Figure 6D, top). Reassuringly, codepletion of PARP1 again completely abolished SAFB1 recruitment (Figure 6D, bottom), suggesting that PARP1, rather than PARP2 or any other PARP family member, is mainly responsible for the observed SAFB1 recruitment. Efficient PARP1 and PARP2 depletion was confirmed by western blot analysis (Figure S6C); the effect of PARG depletion was indirectly evident by increased PARP1 autoPARylation after PARG knockdown (Figure S6C). Our findings are consistent with previous data showing that PARP1 has higher intrinsic catalytic activity than other PARPs (Altmeyer et al., 2009) and with PARP1 being responsible for the largest part of PARylation in response to DNA damage.

Next, we analyzed the detailed kinetics of SAFB1 recruitment and exclusion live with ectopically expressed GFP-SAFB1 in cells depleted of PARG and treated with Mirin. These experiments consistently revealed almost immediate SAFB1 dissociation followed by a remarkable recruitment to the borders of the damaged chromatin domain, which was eventually followed by persistent release from the damaged area (Figures 6E and S6D

(F) Cells were treated with IR as indicated and stained for 53BP1 and SAFB1.

(G) Cells were stained for 53BP1 and SAFB1 in order to monitor SAFB1 exclusion from 53BP1 bodies marking spontaneous DNA damage in G1 cells. Scale bars represent 10 μm.

See also Figure S5 and Movie S1.

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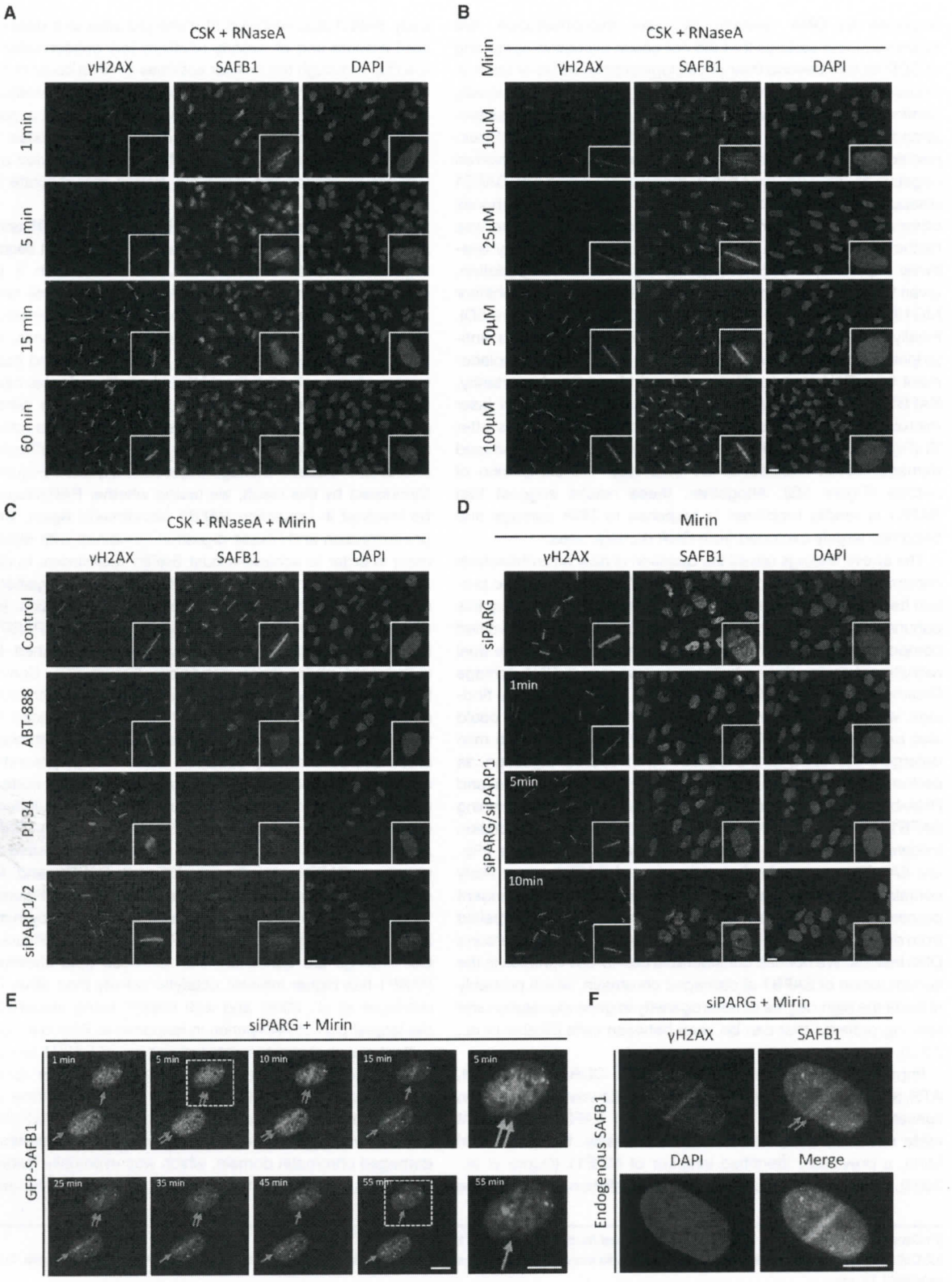

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and Movie S2). Importantly, this unusual transient recruitment to the rims of the modified chromatin domain could also be observed for endogenous SAFB1 (Figure 6F). The accumulation of SAFB1 specifically at the borders of damaged nuclear areas was neither caused by excessive nucleosome eviction in the center of the modified domain, given that not only NBS1 and γH2AX but also the nucleosome-binding genome caretaker 53BP1 accumulated readily at the center of the damaged chromatin domain under these conditions (Figures S6D and S6E), nor was it caused by excessive DNA end resection, as revealed by a normal accumulation of the single-stranded DNA-binding protein RPA (Figure S6F). Furthermore, consistent with PARP1- and PAR-dependent recruitment, the inhibition of the DDR kinases ATM, ATR, and DNA-PK or depletion of RNF8 and RNF168 did not abolish SAFB1 recruitment under these conditions (Figure S6G). PARG depletion alone resulted in a weak accumulation of SAFB1 around damaged chromatin of both endogenous SAFB1 (Figure S7A) and GFP-SAFB1 (Movie S3). Increased chromatin acetylation by TSA treatment had only a minor effect on SAFB1 recruitment in PARG-depleted, Mirin-treated cells (Figures S7B and S7C and Movie S4). However, the addition of the PARP inhibitor ABT-888 (veliparib) completely abolished SAFB1 accumulation even after PARG depletion and in the presence of Mirin (Figure S7D and Movie S5), thus confirming that SAFB1 recruitment is strictly dependent on PAR formation. Importantly, we also observed SAFB1 accumulation in the absence of Mirin when we codepleted PARG and DNA Ligase IV, an essential component of the nonhomologous end-joining pathway (Figure S7E and Movie S6). Collectively, we conclude that, by slowing down DNA repair initiated immediately after break induction and concurrently enhancing the half-life of DNA-damage-induced PARylation, the fraction of SAFB1 that is recruited to damaged chromatin exceeds the fraction of SAFB1 potentially involved in transcription or splicing, therefore allowing for direct visualization of this PAR-responsive protein pool.

We followed cells with transient SAFB1 recruitment live for up to 18 hr without observing signs of nuclear disintegration (Movie S7), indicating that the PAR-dependent SAFB1 recruitment is not an early response to the induction of cell death but rather reflects a repair-associated chromatin response to damage induction. Finally, mutational analysis of SAFB1 revealed that a carboxyl terminal arginine- and glycine-rich domain containing an RGG motif of the form RGGMSGRG was required and sufficient for SAFB1 recruitment (Figure 7A), suggesting that this unstructured low-complexity region of SAFB1 mediates its PARP1- and PAR-dependent accumulation at damaged chromatin (see also the Discussion). We also found that the transient recruitment of SAFB1 was markedly different from previously reported recruitment and exclusion dynamics of hnRNP-like proteins (Polo et al., 2012); i.e., SAFB1 exclusion was still observed under conditions where hnRNPUL1 was recruited to damaged chromatin (for instance, upon high laser power [Figure S7F] or chemical inhibition of transcription [Figure S7G]), thus indicating that protein exclusion is not simply a consequence of disassembly and removal of the RNA-polymerase II-associated transcription machinery. On the contrary, these data indicate that, although SAFB1 recruitment may assist the early chromatin response to DNA breakage, its subsequent exclusion might help prevent excessive signaling. To approach this idea experimentally, and in an attempt to extend SAFB1's engagement with the damaged chromatin, we fused SAFB1 to the minimal focus-forming domain (FFD) of the genome caretaker 53BP1 (Figure 7B). Unlike wild-type (WT) SAFB1, the GFP-SAFB1-FFD fusion protein was readily detectable at sites of spontaneous DNA damage (Figure 7C), at IR-induced foci (Figure 7D), and at laser-microirradiation-induced DNA breaks (Figure 7E). When compared to cells expressing similar amounts of GFP-FFD, as assessed by automated quantitative high-content image analysis, GFP-SAFB1-FFD-expressing cells had elevated levels of γH2AX, suggesting that enforced SAFB1 presence at damaged chromatin can lead to enhanced H2AX phosphorylation (Figure 7F). Stimulated by this result, we tested whether the overexpression of WT SAFB1 would also increase γH2AX spreading in response to DNA damage. When cells were gated for different levels of GFP expression, SAFB1-GFP-expressing cells showed higher γH2AX levels in comparison to GFP-expressing cells (Figure 7G) consistently and in a manner that correlated with expression levels. Altogether, these results suggest that the enforced engagement of SAFB1 with damaged chromatin causes excessive γH2AX spreading.

DISCUSSION

In this study, we identified the scaffold attachment factor SAFB1 as an architectural chromatin component of the cellular response to genotoxic stress. SAFB1 is transiently recruited to DNA damage sites in a PARP1- and PAR-dependent manner and is required for efficient signaling and the spreading of chromatin phosphorylation in response to genotoxic stress. SAFB1 seems to cooperate with chromatin acetylation and counteract chromatin insulators in order to render chromatin permissive for DNA damage signaling. Our data suggest that the early recruitment of chromatin components such as SAFB1 is needed

Figure 6. SAFB1 Is Transiently Recruited to Damaged Chromatin in a PARP1- and PAR-Dependent Manner

(A) After laser microirradiation, cells were pre-extracted as indicated and stained for γH2AX and SAFB1.

(B) After laser microirradiation in the presence of increasing doses of Mirin added directly before damage induction, cells were pre-extracted as indicated and stained for γH2AX and SAFB1.

(C) Cells were cotreated with the PARP inhibitors ABT-888 and PJ-34 or codepleted of PARP1 and PARP2 as indicated. Then, cells were laser microirradiated and stained for γH2AX and SAFB1.

(D) Cells were treated as indicated and stained for γH2AX and SAFB1.

(E) GFP-SAFB1-expressing cells were followed live after laser microirradiation in order to reveal recruitment to the rims of the modified chromatin domain.

(F) Cells were treated as indicated and stained for γH2AX and SAFB1 in order to reveal the recruitment of endogenous SAFB1 to the rims of the modified chromatin domain. Scale bars represent 10 μm.

See also Figures S6 and S7 and Movies S2–S7.

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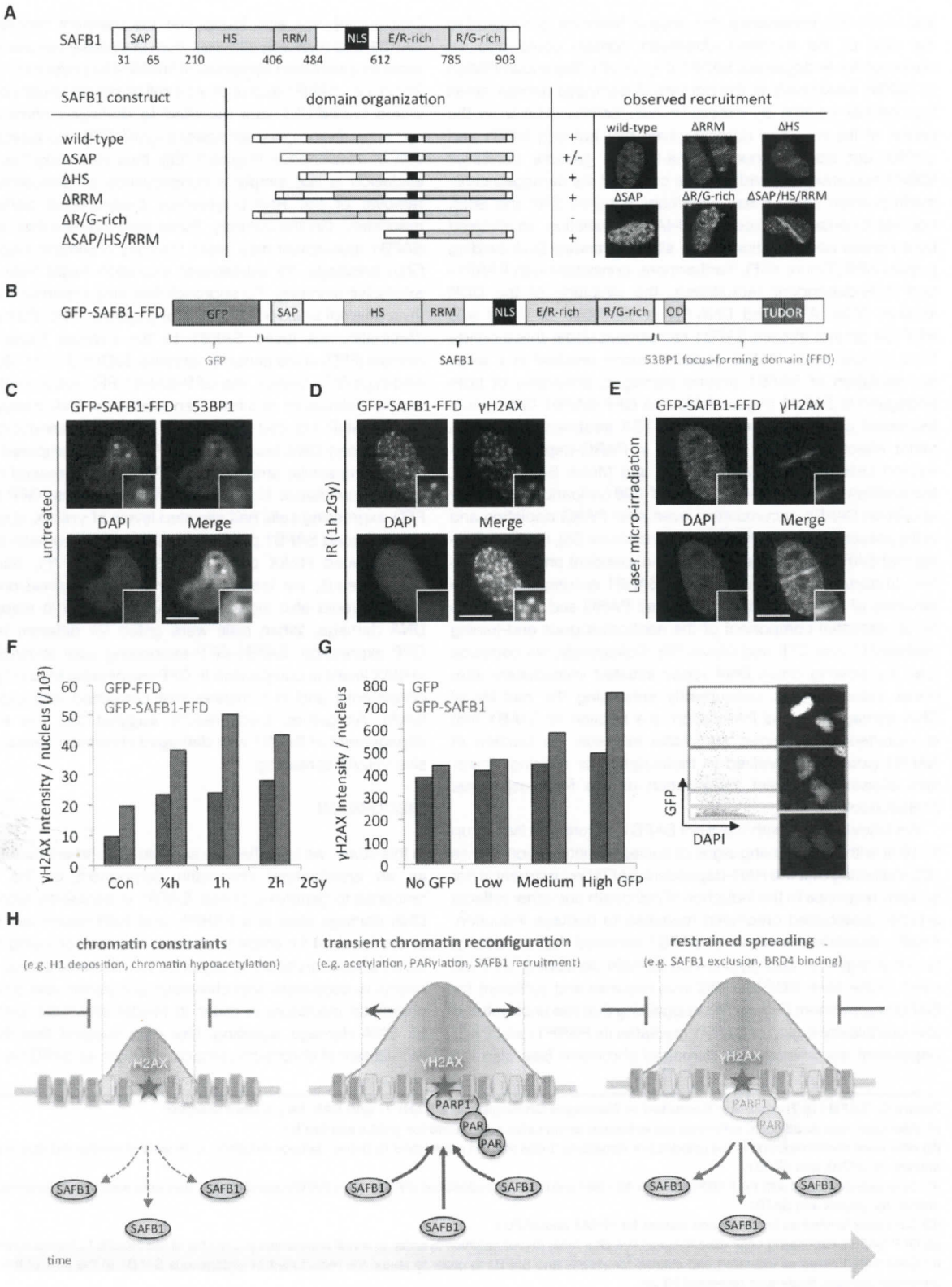

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in order to overcome physiological barriers imposed by the chromatin landscape, which most likely evolved to guard against unwarranted or excessive DNA damage signaling. For instance, it was recently reported that the bromodomain protein BRD4 and the cohesin and condensin complexes antagonize γH2AX spreading (Caron et al., 2012, Floyd et al., 2013). Thus, although chromatin insulators may act to locally constrain H2AX phosphorylation, the transient recruitment of SAFB1 could provide a window of opportunity to reach the threshold required to initiate a timely and robust DDR. Once an efficient response has been set in motion, SAFB1 is released and eventually excluded from damaged chromatin, possibly for preventing unwanted excessive chromatin transactions (Figure 7H), and it could be that the multitude of DNA-damage-induced modifications identified on SAFB1 (Beli et al., 2012; Bennetzen et al., 2010; Bensimon et al., 2010) cooperate to release, and then exclude, SAFB1 from damaged chromatin.

Importantly, the function of SAFB1 in assisting the chromatin response to genotoxic stress described herein is essential for properly maintaining replication fork stability and cell-cycle checkpoint control, both of which are important cellular features that are often subverted in human cancers. In this regard, our data strongly reinforce the notion that relatively mild defects in DDR signaling can severely undermine genome integrity. Given that endogenous replication stress and the generation of DSBs are common features of cancer development, to which the DDR serves as intrinsic barrier (Jackson and Bartek, 2009), the frequently observed loss of SAFB1 in human tumors might represent one way that cancer cells escape genome surveillance mechanisms. In light of the pronounced sensitivity of SAFB1-depleted cells to replication-stress-induced DNA damage, it could be that the subset of tumors that lost SAFB1 are sensitive to treatment with drugs that cause replication-associated DNA damage.

The dynamic exchange of SAFB1, including its early PARP1-mediated recruitment followed by sustained exclusion, appears independent of and qualitatively different from previously reported cases of dual recruitment and dissociation behavior (Adamson et al., 2012; Polo et al., 2012), thus indicating that SAFB1 might represent an uncharacterized class of architectural chromatin components that rapidly relocalize in response to DNA damage. By analogy to the intensively studied accumulation of genome caretakers and chromatin modifiers at damaged chromatin and into irradiation-induced foci, which comprises various distinct recruitment mechanisms and different recruitment kinetics and residence times and involves many different proteins with highly specific functions, the newly discovered dual recruitment and exclusion behavior could represent an equally fundamental phenomenon and also comprise equally diverse regulatory mechanisms and functions. In other words, although early and transient recruitment followed by sustained exclusion may turn out to be an emerging general theme for the chromatin response to DNA damage, the recruitment modalities and the functions of the proteins involved—RBMX (Adamson et al., 2012), hnRNPUL1/2 (Polo et al., 2012), SAFB1 (this study), and potentially others—could be highly diverse, and the mechanisms involved in their dissociation could be as complex as the intensely studied mechanisms of protein accumulations at damaged chromosomes. Furthermore, there could be hitherto unappreciated crosstalk among these early responding factors that might determine the dynamic formation of DNA-damage-induced nuclear compartments.

SAFB1 recruitment to damaged chromatin was dependent upon PARP1 and chromatin PARylation, which, aside from having important functions in various stress signaling pathways (Altmeyer and Hottiger, 2009; Bürkle and Virág, 2013; Kraus and Hottiger, 2013), is one of the earliest and most transient responses to DNA breakage. Although the exact function of transient PAR formation at DSBs and its impact on pathway choice and repair is still incompletely understood and most likely complex, a role in assisting the chromatin response to DNA breakage is suggested by the increasing number of chromatin remodelers and genome caretakers that are recruited to break sites in a PAR-dependent manner, one of the latest examples being the BRCA1-BARD complex, whose early recruitment to DSB sites appears to be PAR-dependent (Li and Yu, 2013). Besides their role in DSB repair, PARP1 and chromatin PARylation have also been implicated in the cellular response to replication stress (Bryant et al., 2009; O'Neil et al., 2013), and PARP inhibitors were shown to sensitize cells to HU, topoisomerase poisons, and alkylating agents (O'Neil et al., 2013). Therefore, we propose that PARylation functions at least in part by transiently recruiting SAFB1 to render chromatin permissive for DNA damage signaling. In line with this notion, it could be that one of the facets of how PARP inhibitors work is by blocking SAFB1 function, which, in turn, might result in an accumulation of endogenous DNA damage and, eventually, cell death.

Recent proteome-wide analyses of PAR-binding proteins revealed that many nucleic-acid-binding proteins, including various mRNA splicing factors, hnRNPs, and hnRNP-like proteins, also have affinity for PAR despite the fact that they lack

Figure 7. Enforced SAFB1 Accumulation at Damaged Chromatin Enhances γH2AX Formation

(A) Domain organization of SAFB1 and observed recruitment of the indicated SAFB1 mutants to laser damage.

(B) Domain organization of GFP-SAFB1 fused to the minimal focus-forming domain (FFD) of 53BP1.

(C) Cells were transfected with GFP-SAFB1-FFD and costained for 53BP1.

(D) Cells were transfected with GFP-SAFB1-FFD, irradiated as indicated, and costained for γH2AX.

(E) Cells were transfected with GFP-SAFB1-FFD, laser microirradiated, fixed after 10 min, and costained for γH2AX.

(F) Cells were transfected with GFP-FFD or GFP-SAFB1-FFD and irradiated as indicated, and cells expressing matched high levels of GFP were analyzed for nuclear γH2AX intensity by automated high-content imaging.

(G) Cells were transfected with GFP or GFP-SAFB1, irradiated with 1 Gy, and fixed after 15 min, and cells expressing matched levels of GFP were analyzed for nuclear γH2AX intensity by automated high-content imaging.

(H) A model depicting dynamic SAFB1 recruitment and release. Transient PARP1- and PAR-dependent recruitment of SAFB1 can help overcome physiological barriers imposed by chromatin hypoacetylation, hypercondensation, or insulation. Scale bars represent 10 μm.

specific PAR-binding motifs (Krietsch et al., 2012). Therefore, it is tempting to speculate that transient, local PAR formation is a general mechanism for outcompeting RNA binding and thereby providing a trigger and nucleation event to directly recruit proteins from their normal sites of nuclear residency into dynamic, self-assembly-assisted aggregates to function in the DDR. Depending on their original and potentially cell-type-specific function, the recruited proteins may then help to reorganize higher-order chromatin structure, sequester or actively remove nascent, unprocessed RNAs, block components of the transcription elongation complex, regulate alternative splicing, prevent the formation of or resolve R loops, or even induce the expression of noncoding RNAs. An intriguing and hitherto unappreciated alternative possibility is that local PAR formation could counteract the dissociation of chromatin components that might be displaced by the physical forces associated with topological changes brought about by disrupting the DNA double helix that is inevitably under tension. Although additional work is required to investigate such possibilities in more detail, in this study on PAR-dependent recruitment of the scaffold attachment factor B1, we highlight one example of how delicately balanced chromatin dynamics impact on DDR signaling to prevent genomic instability.

EXPERIMENTAL PROCEDURES

Human U-2-OS osteosarcoma cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Gibco). siRNA transfections were performed for 72 hr with Lipofectamine RNAiMAX (Invitrogen) and 25 nM Ambion Silencer Select siRNA duplexes. Plasmid transfections were performed for 24 hr with Lipofectamine LTX and PLUS Reagent (Invitrogen). Immunostainings and immunochemical assays were performed according to standard procedures. Automated multichannel wide-field microscopy for high-content imaging of asynchronous cell populations was performed on an Olympus ScanR system as previously described (Gudjonsson et al., 2012). Laser microirradiation experiments were performed on BrdU presensitized cells with a 355 nm UV-A pulsed laser. Supplemental Experimental Procedures are provided in the Supplemental Information.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, one table, and seven movies and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2013.08.025.

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