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CHAPTER 3

POLY(ADP-RIBOSYL)ATION LINKS THE CHROMATIN REMODELER SMARCA5/SNF2H TO RNF168-DEPENDENT DNA DAMAGE SIGNALING

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ABSTRACT

Ionizing radiation (IR)-induced DNA double-strand breaks (DSBs) in native chromatin elicit cellular responses such as cell cycle checkpoint activation and DNA repair. How cells signal and repair DSBs within chromatin remains enigmatic. Here, we show that SMARCA5/SNF2H, the catalytic subunit of ISWI chromatin remodeling complexes, is recruited to DSBs in a poly(ADP-ribose) polymerase (PARP)-dependent, but ATM/DNA-PK/MDC1-independent manner. Remarkably, we found that PARP activity not only is required for the spreading of SMARCA5, but also affects the distribution of the E3 ubiquitin ligase RNF168, ubiquitin conjugates and the ubiquitin-binders RAD18 and the RAP80-BRCA1 complex into the DSB-flanking chromatin compartment. This suggests that PARP regulates the spatial organization of the RNF168-driven ubiquitin response to DNA damage. In support of this, we show that PARP activity stimulates the DNA damage-induced interaction between SMARCA5 and RNF168, providing a molecular mechanism for how PARP is linked to the RNF168 ubiquitin cascade. Furthermore, we demonstrate that SMARCA5 regulates the ubiquitin response by promoting RNF168 accumulation at DSBs, which subsequently facilitates efficient formation of ubiquitin conjugates and assembly of BRCA1. Underlining the importance of these findings, we show that depletion of SMARCA5 renders cells sensitive to IR and results in DSB repair defects by non-homologous end-joining and homologous recombination. Interestingly, loss of SMARCA5 sensitizes cells to PARP inhibition, suggesting that SMARCA5 is a novel target for PARP inhibitor-based cancer therapies. Our study unveils a physical and functional link between DNA damage-induced poly(ADP-ribosyl)ation, SMARCA5-mediated chromatin remodeling and RNF168-dependent signaling and repair of DSBs.

INTRODUCTION

A major threat to genome stability is the formation of chromosomal DNA double-strand breaks (DSBs), which can be generated by exposure of cells to agents such as ionizing radiation (IR), or arise during DNA replication. Inefficient or inaccurate repair of DSBs can lead to chromosome rearrangements, which may result in cancer development or cell death (Jackson and Bartek, 2009; Negrini et al., 2010). To circumvent the deleterious effects of DSBs, cells activate the DNA damage response (DDR), which comprises events that lead to the detection and repair of these lesions, as well as a delay in cell cycle progression until repair is completed (Huen and Chen, 2010). The repair of DSBs involves two dedicated pathways known as non-homologous end-joining (NHEJ) and homologous recombination (HR). Repair by NHEJ promotes either error free or error-prone ligation of broken DNA ends, whereas HR mediates only error-free repair of DSBs by using the non-damaged sister chromatid as a template (Wyman and Kanaar, 2006).

Numerous DDR proteins accumulate at IR-induced DSBs and form cytologically discernable structures known as ionizing radiation-induced foci (IRIF) (Bekker-Jensen and Mailand, 2010). The initial key step in IRIF formation is the ATM/DNA-PK-mediated phosphorylation of histone variant H2AX (termed γ H2AX). The MDC1 protein subsequently binds to γ H2AX and mediates recruitment of the E3 ubiquitin ligases RNF8 and RNF168 together with the HERC2 protein and the E2 ubiquitin conjugase UBC13. The ensuing chromatin ubiquitylation catalyzed by RNF8/RNF168/HERC2/UBC13 promotes the recruitment of several proteins, including BRCA1 and 53BP1, which culminates in checkpoint activation and DNA repair (Doil et al., 2009; Huen et al., 2007; Mailand et al., 2007; Stewart et al., 2009; Wang and Elledge, 2007; Bekker-Jensen et al., 2010).

Efficient signaling and repair of DSBs is hampered by the fact that genomic DNA is packaged into chromatin through histone and non-histone proteins. Histone proteins are extensively modified in response to DNA damage and it is becoming clear that the crosstalk between histone modifications in DSB-flanking chromatin controls the DDR (van Attikum and Gasser, 2009; Luijsterburg and van, 2011). However, how this is accomplished remains largely elusive. Recent studies have begun to explore this in mammalian cells and demonstrated that the TIP60 and SWI/SNF chromatin remodeling complexes facilitate DSB repair by promoting histone acetylation and H2AX phosphorylation at DSBs, respectively (Murr et al., 2006; Park et al., 2006; Lee et al., 2010). The p400 and CHD4 ATPases, which are catalytic subunits of the TIP60 and NuRD chromatin remodeling complexes, respectively, not only facilitate DSB repair, but also promote the RNF8/RNF168 ubiquitin response at DSBs (Xu et al., 2010; Smeenk et al., 2010; Larsen et al., 2010; Polo et al., 2010). More recently it was found that SMARCA5/SNF2H, which is the ATPase of several distinct ISWI chromatin remodeling complexes involved in transcription and replication, is also involved in the DSB response (Lan et al., 2010; Nakamura et al., 2011; Sanchez-Molina et al., 2011). However, precisely how SMARCA5 regulates signaling and repair of DSBs remains unclear.

Here we demonstrate that distinct ATM/ γ H2AX/MDC1- and poly(ADP-ribose) polymerase (PARP)-dependent pathways lead to recruitment of RNF8/RNF168 and SMARCA5 to DSBs, respectively. SMARCA5 and RNF168 interact in the DSB-containing chromatin compartment in a PARP-dependent manner to regulate histone ubiquitylation, BRCA1 accumulation and DSB repair. Our findings unveil a novel physical and functional link between DNA damage-induced poly(ADP-ribosylation) (PARylation), chromatin remodeling and the RNF8/RNF168-signaling cascade at DSBs.

RESULTS

SMARCA5 protects human cells against IR

A genome-wide RNAi screen in the nematode *Caenorhabditis elegans* identified 45 genes, including known DNA damage response (DDR) factors, as well as several novel genes such as *isw-1*, that protect worms against IR (van Haaften et al., 2006). Database searches revealed two human proteins, SMARCA1/SNF2L (63% identity and 79% similarity) and SMARCA5/SNF2H (64% identity and 79% similarity), which are highly homologous to *C. elegans* ISW-1. Both proteins are members of the ISWI subclass of the SNF2 ATPase family (Bao and Shen, 2011), and are catalytic subunits of ATP-dependent chromatin remodeling complexes involved in transcription regulation and DNA replication (Ito et al., 1999; Poot et al., 2000; LeRoy et al., 2000; Collins et al., 2002; Barak et al., 2003). In this study we focused on SMARCA5 and examined its role in the cellular response to DSBs. Based on the findings in *C. elegans*, we first tested whether knockdown of SMARCA5 affects clonogenic survival of mammalian cells after exposure to IR. In agreement with a previous study (Lan et al., 2010), we found that RNAi-mediated depletion of SMARCA5 led to a marked increase in IR sensitivity comparable with that observed after XRCC4 or ATM knockdown (Fig. 1, a and b; Fig. S1, a and b).

SMARCA5 is required for DSB repair by NHEJ

To assess the involvement of SMARCA5 in the rejoining of IR-induced DSBs, we performed neutral comet analysis. DSB levels were slightly elevated in non-irradiated SMARCA5-depleted cells, suggesting that SMARCA5 may contribute to genomic stability by preventing spontaneous chromosome breakage. Importantly, irradiation of cells with IR resulted in comparable numbers of DSBs in SMARCA5-depleted cells and control cells (Fig. S1, c and d; 0 h time point). Conversely, the amount of DSBs was significantly higher at 2 h after IR in SMARCA5-knockdown cells compared to control cells, but nearly returned to levels observed in control cells at 6 h (Fig. S1, c and d). Thus, loss of SMARCA5 may attenuate DSB repair and delay cell cycle progression. Indeed, SMARCA5-depleted cells accumulated in the G2 phase after IR, but, in contrast to control cells, failed to recover from this arrest (Fig. S1, e and f). These results suggest that SMARCA5 promotes efficient repair of DSBs.

To further assess the role of SMARCA5 in DSB repair we employed the EJ5-GFP reporter system, which measures repair between two tandem I-SceI sites that separate a *GFP* gene from its promoter. Following I-SceI expression, repair of the two I-SceI-induced DSBs by NHEJ fuses the promoter to the *GFP* coding sequence and restores GFP expression (Fig. S2 a) (Bennardo et al., 2008). As expected, loss of the NHEJ factor XRCC4, but not of the HR factor BRCA2, reduced the efficiency of NHEJ as measured by GFP fluorescence (Fig. 1 c). Interestingly, SMARCA5 depletion impaired NHEJ to the same extent as XRCC4 knockdown (Fig. 1 c). Cell cycle profiles were not altered in these cells, ruling out any effect of changes in the cell cycle on the efficiency of NHEJ (Fig. S2 c). Thus, our results strongly suggest that SMARCA5 promotes DSB repair by NHEJ.

SMARCA5 is required for DSB repair by HR

Next we determined the role of SMARCA5 in HR by using the DR-GFP reporter system. In this system, a single I-SceI site is present in one of two defective GFP reporter genes. Following I-SceI expression, repair of the I-SceI-induced DSB by gene conversion produces a functional *GFP* gene. (Fig. S2 b) (Weinstock et al., 2006). As expected, depletion of BRCA2 dramatically reduced the HR efficiency (Fig. 1 d) (Moynahan et al., 2001). Remarkably, a similar reduction in HR repair was observed after SMARCA5 depletion (Fig. 1 d). As cell cycle profiles remained unchanged in these cells, we can rule out that changes in the cell cycle affected the efficiency of HR (Fig. S2 c). Thus, our results suggest that SMARCA5 is required for DSB repair by HR. Previous work has implicated SMARCA5 in DNA damage repair (Lan et al., 2010; Nakamura et al., 2011). Our results, which demonstrate that SMARCA5 regulates the two major pathways of DSB repair (NHEJ and HR) are fully consistent with these observations.

SMARCA5 protects cells against the genotoxic effects of PARP inhibition

PARP is a key factor in the single-strand break repair pathway. If PARP is inhibited, single-strand breaks remain unrepaired and may be converted to DSBs during DNA replication. Consequently, PARP inhibition is selectively lethal to cells deficient for factors involved in HR (McCabe et al., 2006). To confirm the role of SMARCA5 in HR, we tested whether SMARCA5 depletion would also render cells sensitive to PARP inhibition. Clonogenic survival assays showed enhanced sensitivity of SMARCA5-depleted cells to PARP inhibition, which paralleled the sensitivity of BRCA2-depleted cells (Fig. 1 e). These findings are consistent with our previous results using the DR-GFP reporter (Fig. 1 d), and support a role for SMARCA5 in DSB repair by HR.

SMARCA5 is rapidly recruited to DSBs

Recent work reported that SMARCA5 is recruited to sites of DNA damage (Lan et al., 2010; Nakamura et al., 2011; Erdel et al., 2010; Sanchez-Molina et al., 2011). We confirmed these results by using a U2OS cell line expressing a 4-hydroxytamoxifen (4-OHT)-inducible

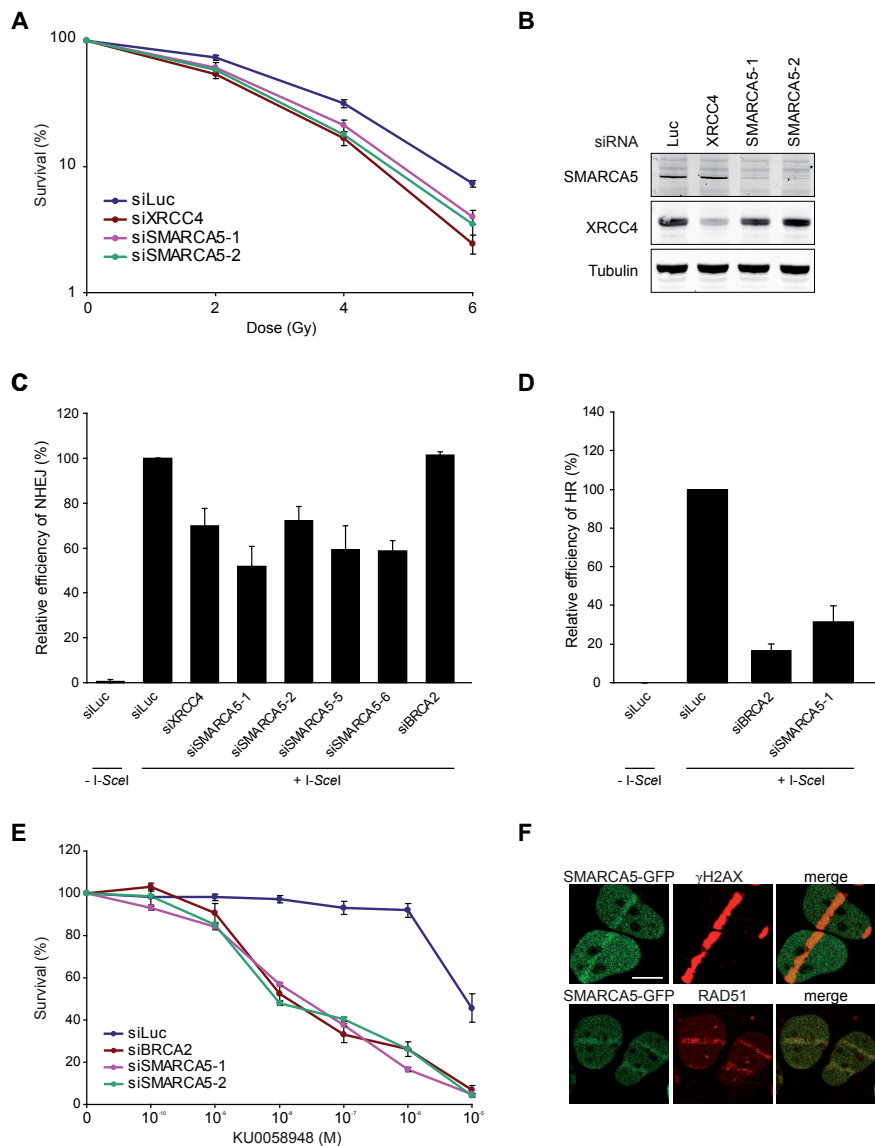


Figure 1. SMARCA5 protects cells against IR and is recruited to DSBs to promote DNA repair. (A) VHI10-SV40 cells were transfected with the indicated siRNAs, exposed to IR and scored for clonogenic survival. Graphs represent the mean $\pm$ s.e.m. of 3 independent experiments. (B) SMARCA5 and XRCC4 levels were monitored by western blot analysis using whole cell extracts (WCE) of cells in A. Tubulin is a loading control. (C) HEK293T cells containing the NHEJ reporter EJ5-GFP were transfected with the indicated siRNAs and 48 h later transfected with an I-SceI expression vector (pCBASce). The mean $\pm$ s.e.m. of 3 experiments is shown. (D) As in C, except that cells containing the HR reporter DR-GFP were used. (E) As in A, except that cells were treated with PARP inhibitor (KU0058948). (F) U2OS cells expressing SMARCA5-GFP were subjected to multiphoton laser irradiation. After 15 min cells were immunostained for γ H2AX and RAD51.

restriction enzyme, *Asi*SI-ER, which induces site-specific DSBs as evidenced by the formation of γ H2AX and MDC1 foci (Fig. S3, a and b) (Iacovoni et al., 2010). Chromatin immunoprecipitation (ChIP) coupled to quantitative PCR (qPCR) detected 4-OHT-induced γ H2AX and SMARCA5 accumulation at two different sites flanking *Asi*SI-induced DSBs on chromosome 1 and 22 (Fig. S3, c and d). Thus, SMARCA5 accumulates at DSBs, which suggest that SMARCA5 is directly involved in DSB repair, agreeing with recent studies (Lan et al., 2010; Nakamura et al., 2011; Erdel et al., 2010).

The kinetics of SMARCA5 recruitment to DNA strand breaks have not been studied and compared to the rate of accumulation of other factors involved in the DSB response. Given that this may provide insight into its role in the DSB response, we examined the assembly kinetics of SMARCA5 at laser-induced DNA damage. GFP-tagged SMARCA5 accumulated in multiphoton laser-induced tracks and spanned the entire chromatin region marked by γ H2AX or RAD51 (Fig. 1 f). Notably, XPA, a nucleotide excision repair protein that recognizes UV-induced DNA lesions, did not accumulate, suggesting that the applied laser settings primarily create DNA strand breaks (Fig. 2 a). SMARCA5 immediately accumulated after irradiation, reaching half-maximum around 40 seconds and maximum levels at 3 - 5 minutes, and remained associated with the DSB-containing chromatin compartment for at least 2 hours (Fig. 2 a; Fig. S3 e). Next, we compared SMARCA5 accumulation to that of the MDC1 and 53BP1 proteins, which assemble "early" and "late", respectively, at laser-induced DSBs (Mailand et al., 2007). Our kinetic studies not only corroborated these findings for MDC1 and 53BP1, but also show that SMARCA5-GFP accumulates at the DSB-track with nearly similar kinetics to GFP-MDC1, suggesting that it is among the "early" proteins that associate with DSBs (Fig. 2, a and b).

PARP CONTRIBUTES TO SMARCA5 RECRUITMENT TO DSBs

Our work and that of several other laboratories demonstrated that SMARCA5 is recruited to sites of laser-induced DNA damage (Lan et al., 2010; Erdel et al., 2010; Sanchez-Molina et al., 2011). A role for the RNF20 E3 ubiquitin ligase in the recruitment of SMARCA5 was reported, yet it remained unclear whether other factors regulate SMARCA5 assembly at DNA strand breaks (Nakamura et al., 2011). The similarity in recruitment kinetics between SMARCA5 and MDC1 suggested to us that MDC1 may play a role in the accumulation of SMARCA5 at laser-induced DNA damage. However, neither depletion of MDC1, nor depletion of RNF168, which is recruited to DNA damage sites in an MDC1-dependent manner, affected SMARCA5 assembly at DSB-tracks (Fig. 2, c and d), suggesting that the MDC1/RNF8/RNF168 signaling cascade does not contribute to SMARCA5 recruitment.

The ATM and DNA-PK kinases play a key role in the signaling and repair of DSBs (Jackson and Bartek, 2009; van Attikum and Gasser, 2009; Luijsterburg and van, 2011).

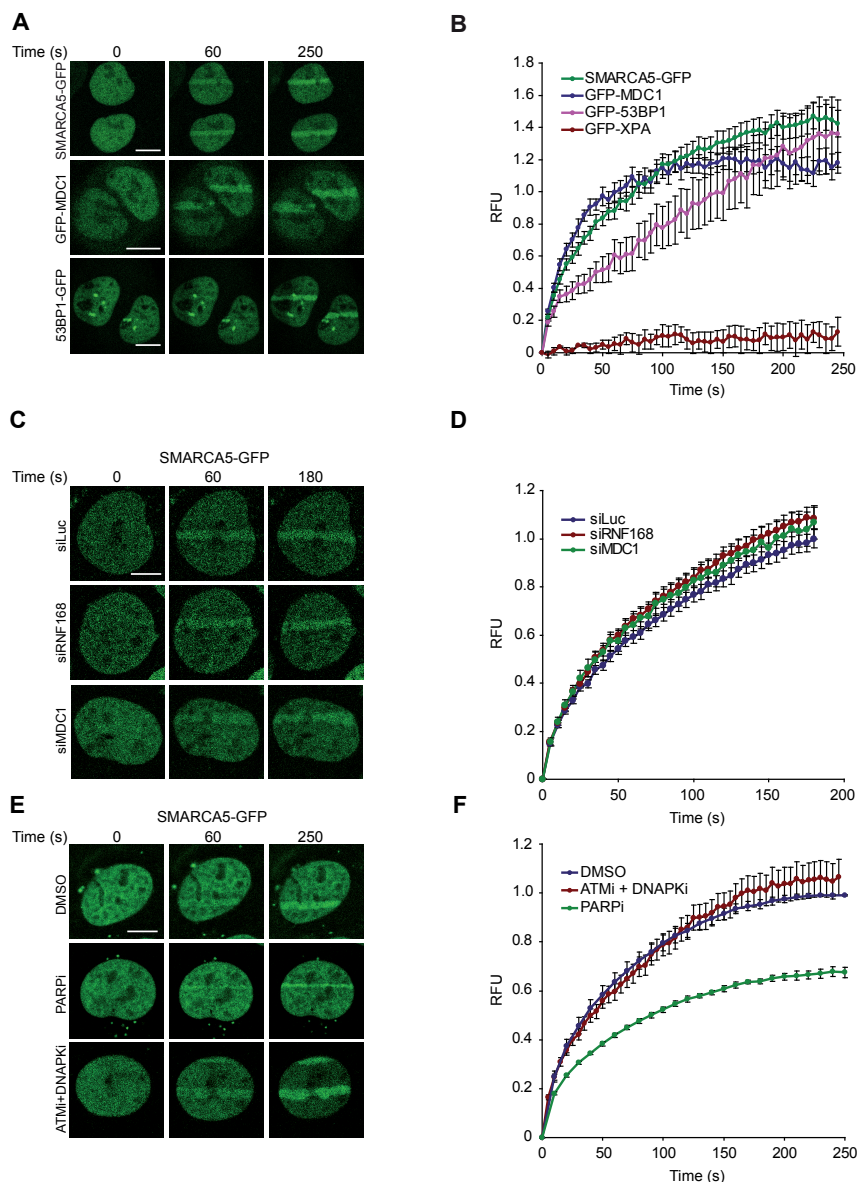


Figure 2. SMARCA5 accumulation at laser-induced DNA damage requires PARP. (A) U2OS cells expressing SMARCA5-GFP, GFP-MDC1, 53BP1-GFP or XPA-GFP (images not shown) were laser-irradiated and subjected to real-time recording of protein assembly at the damaged area. (B) Quantitative representation of results in A. Relative Fluorescence Units (RFU) are plotted on a time scale. Graphs represent the mean $\pm$ s.e.m. of at least 10 individual cells from independent experiments. Scale bars, 10 μ m. (C) As in A, except that U2OS cells expressing SMARCA5-GFP were transfected with siRNAs against MDC1 or RNF168. (D) Quantitative representation of results in C. (E) As in A, except that U2OS cells expressing SMARCA5-GFP were treated with inhibitors of PARP or ATM and DNA-PK. (F) Quantitative representation of results in E. Scale bars, 10 μ m.

We therefore tested whether their activity might contribute to the accumulation of SMARCA5 at DSBs. However, we found that chemical inhibition of ATM and DNA-PK did not affect SMARCA5 assembly in DSBs-tracks (Fig. 2, e and f).

Recently, PARylation by PARP, which binds to DNA strand breaks, was shown to direct the recruitment of the chromatin remodeling enzymes ALC1 and CHD4 to DSBs (Polo et al., 2010; Gottschalk et al., 2009; Chou et al., 2010; Ahel et al., 2009). We therefore examined the contribution of PARP to the recruitment of SMARCA5 to DSBs. Interestingly, the accumulation of SMARCA5 in DSB-tracks was significantly reduced, but not abolished, in cells treated with PARP inhibitor (Fig. 2, e and f). These results suggest that PARP promotes SMARCA5 recruitment to DNA strand breaks.

PARP regulates the occupancy of SMARCA5 and factors of the RNF168 response throughout damaged chromatin

When we examined the effect of PARP inhibition on SMARCA5 accumulation at sites of laser-induced DNA damage, we noticed that DSB-tracks containing SMARCA5-GFP were much more narrow in the presence of PARP inhibitor (Fig. 2 e; Fig. 3, a and b), suggesting that SMARCA5 failed to spread into the DSB-flanking chromatin compartment, a feature that is typically observed for a large number of repair factors (Bekker-Jensen et al., 2006). It was previously shown that DNA damage induced by laser micro-irradiation rapidly triggers local chromatin expansion *in vivo* (Kruhlak et al., 2006), and that PARylation of nucleosomes triggers chromatin expansion *in vitro* (Poirier et al., 1982). These findings prompted us to test whether PARP plays a role in the expansion process at sites of DNA damage and whether this event regulates the spreading of repair factors into the DSB-flanking chromatin compartment. To this end, we inflicted laser-generated DSBs in the presence or absence of PARP inhibitor and subsequently quantified the distribution of a large number of signaling and repair factors in the laser tracks. We found that after irradiation the width of laser tracks containing either MDC1, RNF8, RNF168 or SMARCA5 significantly increased with time, indicating expansion of the damaged chromatin (Fig. S4 b). Strikingly, while the expansion of SMARCA5-GFP was dramatically affected by PARP inhibition, the expansion of endogenous MDC1 in the same cells was hardly affected (Fig. S4 a). We confirmed that the expansion of GFP-MDC1 and that of its binding partner GFP-RNF8 were also only minimally affected by PARP inhibition (Fig. 3, a and b; Fig. S4 b). Since MDC1 and RNF8 physically associate with γ H2AX (Huen et al., 2007; Kolas et al., 2007; Mailand et al., 2007; Stucki et al., 2005), a constituent of damaged chromatin, our results suggest that chromatin expansion at sites of DNA damage is modestly affected by PARP inhibition, while the expansion of SMARCA5 is severely reduced.

Surprisingly, while RNF8 expansion was not affected by PARP inhibitors, we found that expansion of GFP-RNF168 was significantly impaired after PARP inhibition (Fig. 3, a and b; Fig. S4b). RNF168 is an E3 ubiquitin ligase that acts with E2 ubiquitin-conjugating enzyme UBC13 to promote ubiquitin conjugation at DSB sites (Huen et al., 2007; Kolas et al., 2007; Mailand et al., 2007; Stucki

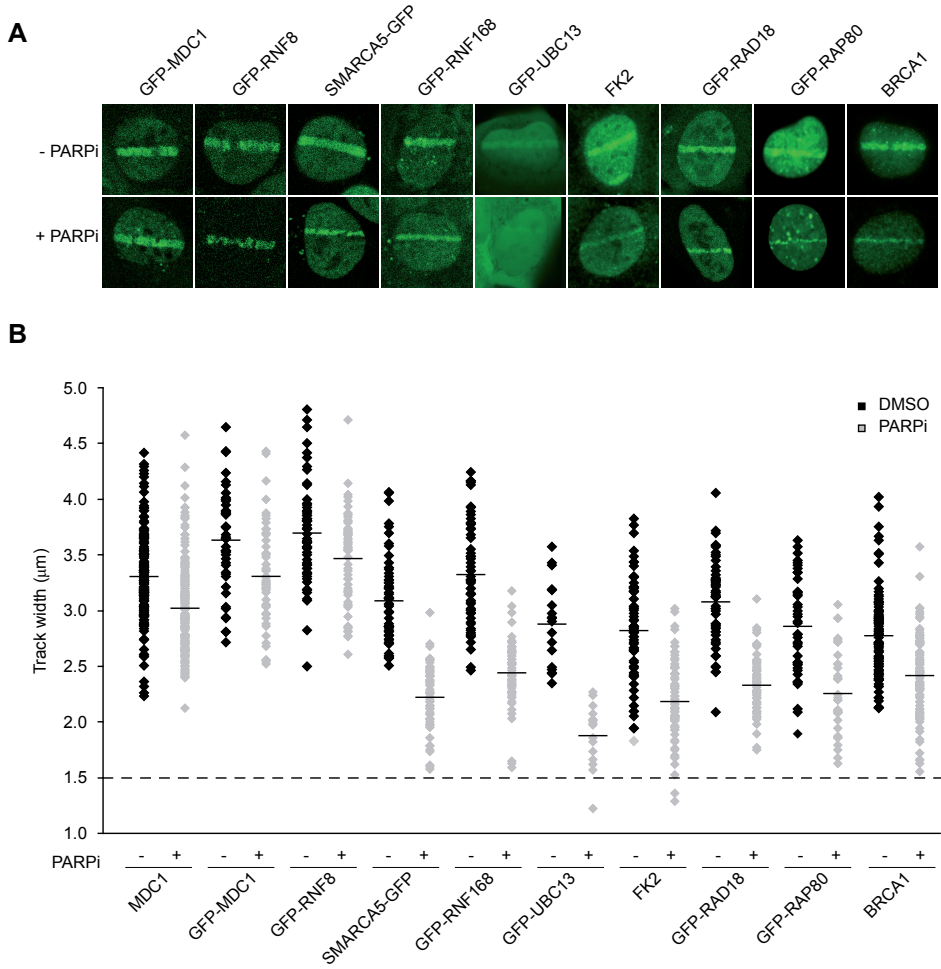


Figure 3. PARP distributes SMARCA5 and factors of the RNF168 cascade throughout damaged chromatin. (A) U2OS cells were treated with PARP inhibitor or left untreated, then subjected to multi-photon laser-irradiation and analyzed for the expansion of the indicated GFP-fusion proteins or immunostained endogenous proteins throughout the damaged compartment. Representative images illustrating the effect of PARP inhibition of the expansion of the indicated proteins are shown. Scale bar, 10 μm . (B) Quantitative analysis of the width of DSB-containing laser tracks from cells in A. Laser tracks with a width of 1.5 μm were generated. After 180 s the width of the region showing accumulation of the indicated proteins was measured to determine the increase in track width. Individual measurements ($n \geq 16$) and mean values are presented in a track-width distribution plot.

et al., 2005; Doil et al., 2009; Stewart et al., 2009). We therefore examined the spreading of GFP-UBC13 and conjugated ubiquitin into DSB-flanking chromatin and found that the expansion of both factors was significantly impaired after PARP inhibition (Fig. 3, a and b). Ubiquitin conjugates generated by RNF8 and RNF168 mediate the recruitment of the ubiquitin-binding factors RAD18 and the RAP80-BRCA1 complex (Huang et al., 2009; Kim et al., 2007; Sobhian et al., 2007; Wang et al., 2007). Consistent with the observed defects in the spreading of ubiquitin conjugates, we found that PARP inhibition significantly reduced the spreading of GFP-RAD18 as well as that of GFP-RAP80 and endogenous BRCA1 (Fig. 3, a and b). Taken together, our comprehensive analysis suggests that PARP activity selectively contributes to the spreading of SMARCA5 and factors of the RNF168 signaling cascade into DSB-flanking chromatin, whereas it only modestly affects the robust spreading of MDC1 and RNF8. Thus, our findings imply a role for an unanticipated PARP-driven mechanism that regulates the occupancy of SMARCA5 and factors of the RNF168 signaling cascade throughout damaged chromatin.

SMARCA5 and RNF168 interact in damaged chromatin in a PARP-dependent manner

The fact that PARP regulates the spreading of both SMARCA5 and factors of the RNF168 signaling cascade may suggest a potential functional link between SMARCA5 chromatin remodeling and RNF168-dependent signaling of DSBs. To gain mechanistic insight into these events, we considered the possibility that SMARCA5 may interact with RNF168. To test this hypothesis, we undertook a quantitative mass spectrometry screen for RNF168-interacting factors, which revealed a DNA damage-dependent interaction between RNF168 and SMARCA5. The interaction between these factors was stimulated after exposure of cells to IR, but not to UV-C light (Fig. 4 a). Immunoprecipitation followed by western blot analysis confirmed the IR-dependent interaction between endogenous RNF168 and SMARCA5 observed in the mass spectrometry screen (Fig. 4 b). Furthermore, we demonstrated that streptavidin-tagged RNF168 interacts with endogenous SMARCA5 and showed in the reciprocal experiment that GFP-tagged SMARCA5 associates with endogenous RNF168 (Fig. 4, c and d). Because SMARCA5 is recruited to DSBs in a PARP-dependent manner (Fig. 2, e and f), and given that PARP inhibitors selectively affect the spreading of factors into damaged chromatin at the level of RNF168, we investigated whether inhibition of PARP affects the interaction between SMARCA5 and RNF168. Remarkably, we found that the IR-induced interaction between endogenous SMARCA5 and RNF168 was nearly completely abolished after treatment with PARP inhibitor (Fig. 4 e). Together, these results strongly suggest that SMARCA5 and RNF168 interact in the DSB-containing chromatin compartment in a manner that requires the activity of PARP and raise the question whether the physical interaction between RNF168 and SMARCA5 regulates the RNF168-ubiquitin response to DNA damage.

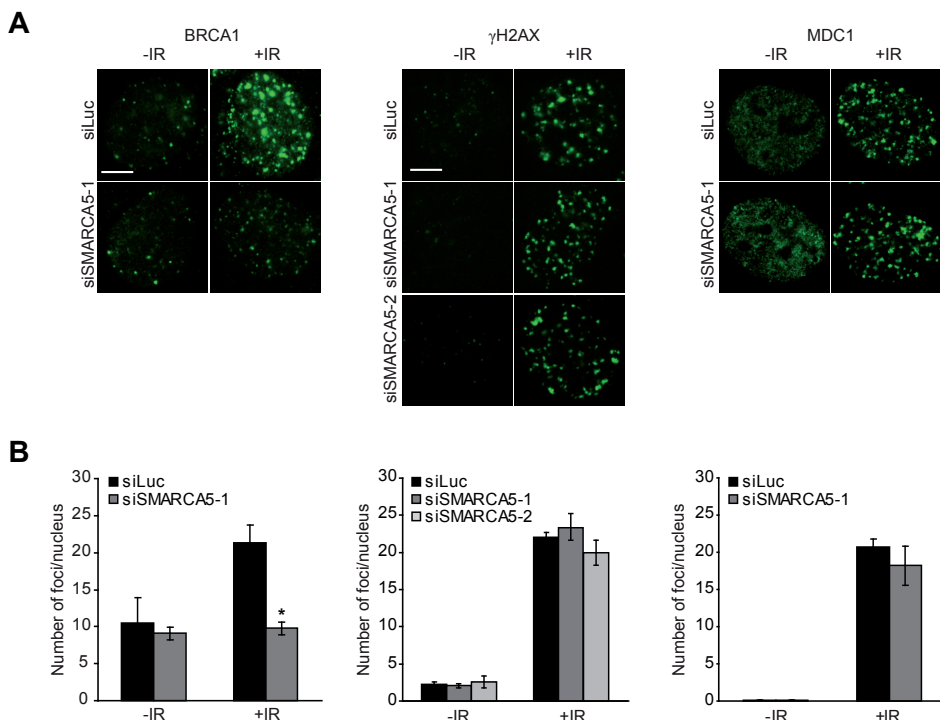


Figure 4. SMARCA5 interacts with RNF168 in a PARP-dependent manner. (A) U2OS cells were SILAC-labeled for 7 days and then mock-treated or exposed to 10 Gy IR or 25 J/m² UV. 1 h later, cells were harvested and subjected to RNF168 immunoprecipitation (IP). Bound complexes were washed, eluted and analyzed by mass spectrometry. Results depict the SILAC ratios measured for the SMARCA5-RNF168 interaction (defined as 1 in the mock sample). (B) HeLa cells were mock-treated or exposed to 10 Gy IR and harvested 1 h later. Whole cell extracts (WCE) were subjected to RNF168 IP followed by immunoblotting for the indicated proteins. (C) HEK293T cells were transfected with Strep-RNF168 plasmid or empty vector and harvested 24 h later. Cell extracts were incubated with Strep-Tactin Sepharose and bound complexes were analyzed by immunoblotting for the indicated proteins. (D) HEK293T cells were transfected with GFP-SMARCA5 plasmid or empty vector and harvested 24 h later. Cell extracts were subjected to GFP IP followed by immunoblotting for the indicated proteins. (E) As in B, except that cells were treated with siRNA against RNF168 or PARP inhibitor (KU0058948).

SMARCA5 stimulates γ H2AX ubiquitylation and BRCA1 accumulation at DSBs

To examine whether SMARCA5 regulates the RNF168 response at DSBs, we examined the impact of SMARCA5 depletion on IRIF formation of several factors involved in the RNF168 signaling cascade. In line with our findings on the spreading of repair factors, we found that SMARCA5 knockdown affected neither γ H2AX and MDC1, nor RNF8 accumulation into IRIF (Fig. 5, a and b). In agreement, western blot analysis showed normal levels of total γ H2AX in these cells after exposure to IR (Fig. S5 a). These results suggest that SMARCA5 does not affect the very early stages of the DSB

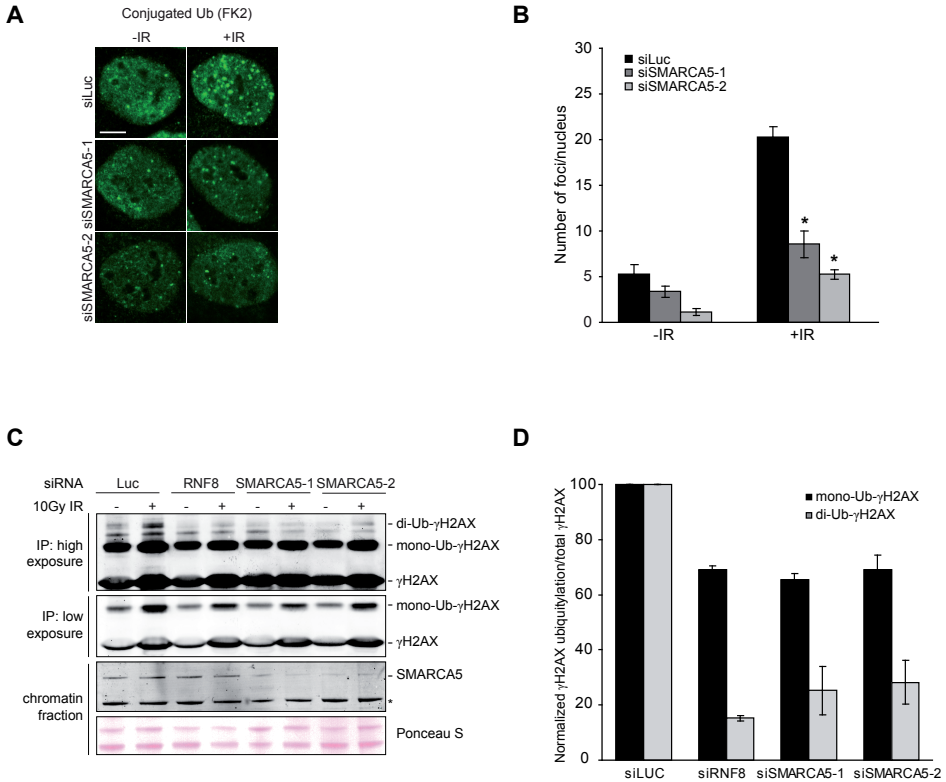


Figure 5. SMARCA5 does not promote γ H2AX, MDC1 or RNF8 accumulation at DSBs. (A) U2OS cells or doxycycline-treated U2OS cells expressing FLAG-RNF8 and an shRNA against RNF8 were transfected with the indicated siRNAs, exposed to 1 Gy IR and 1 h later immunostained for γ H2AX, MDC1 or FLAG-RNF8 to visualize IRIF (Mailand et al., 2007). Scale bar, 10 μ m. (B) Quantitative representation of γ H2AX (left), MDC1 (middle) or FLAG-RNF8 (right) IRIF formation in A. The average number of foci/nucleus $\pm$ s.e.m. is presented for γ H2AX and MDC1, while the percentage of cells with more than 10 foci $\pm$ s.e.m. is presented for FLAG-RNF8. More than 150 nuclei were scored per time point in independent experiments.

signaling response or the initial recruitment of RNF8. In sharp contrast, accumulation of endogenous RNF168 into IRIF was severely impaired by SMARCA5 depletion (Fig. 6, a and b). Corroborating these findings, GFP-RNF168 accumulation in laser tracks was also markedly impaired by depletion of SMARCA5 (Fig. 6, c and d). In agreement with impaired RNF168 recruitment, we found that ubiquitin conjugation in IRIF was impaired in SMARCA5-depleted cells (Fig. 7, a and b). Given that H2A and H2AX are the primary targets for DNA damage-induced ubiquitylation (Doil et al., 2009; Huen et al., 2007; Mailand et al., 2007; Stewart et al., 2009; Wang and Elledge, 2007), we immunoprecipitated γ H2AX from untreated and IR-exposed cells and examined its ubiquitylation status by western blot analysis. In agreement with our foci data,

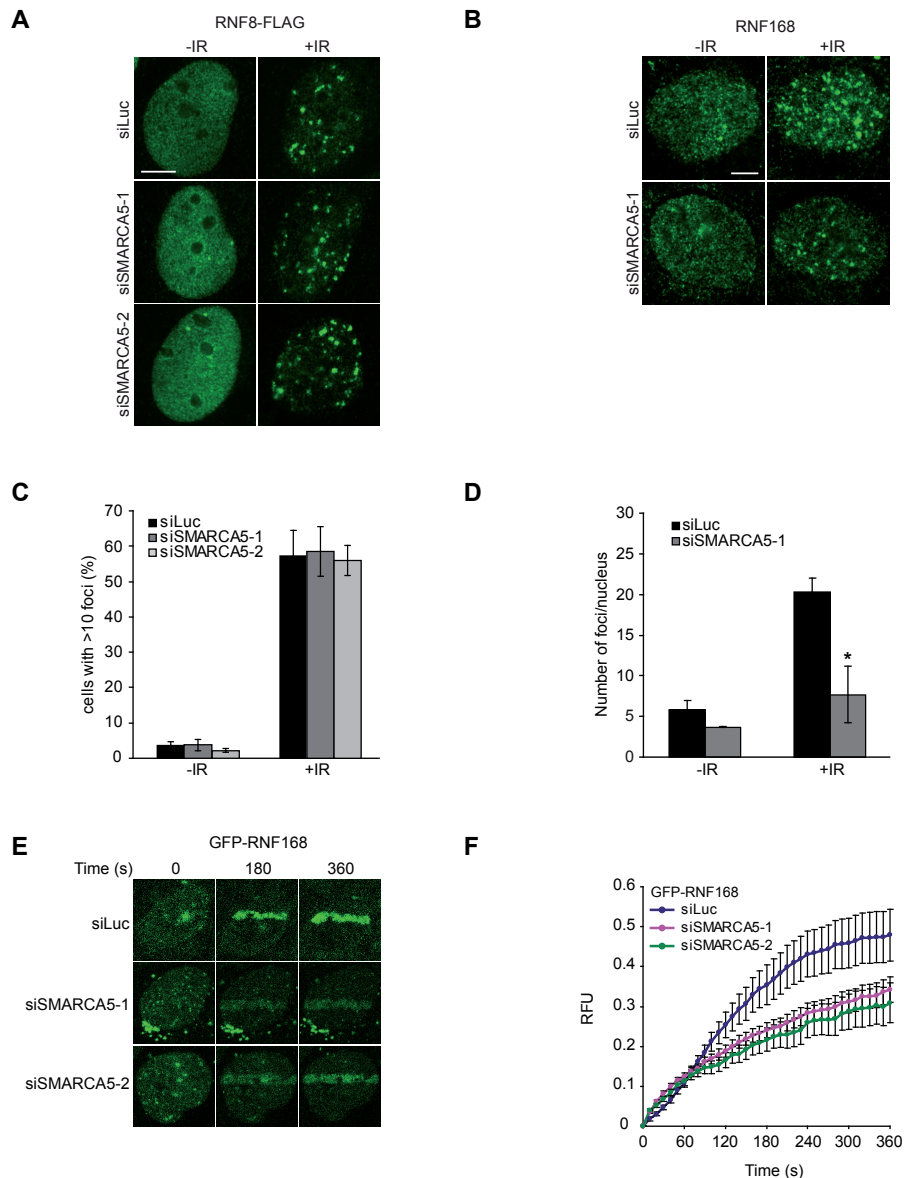


Figure 6. SMARCA5 promotes RNF168 assembly at DSBs. (A) U2OS cells were transfected with the indicated siRNAs, exposed to 1 Gy IR and 1 h later immunostained for RNF168 to visualize IRIF. **(B)** Quantitative representation of RNF168 IRIF formation in A. The average number of foci/nucleus $\pm$ s.e.m. is presented. More than 150 nuclei were scored per time point in independent experiments. *, $P < 0.05$, compared with siLuc (control). **(C)** U2OS cells expressing GFP-RNF168 were transfected with the indicated siRNAs, laser-irradiated and subjected to real-time recording of protein assembly at the damaged area. **(D)** Quantitative representation of results in E. Relative Fluorescence Units (RFU) are plotted on a time scale. Graphs represent the mean $\pm$ s.e.m. of at least 10 individual cells from independent experiments. Scale bars, 10 μ m.

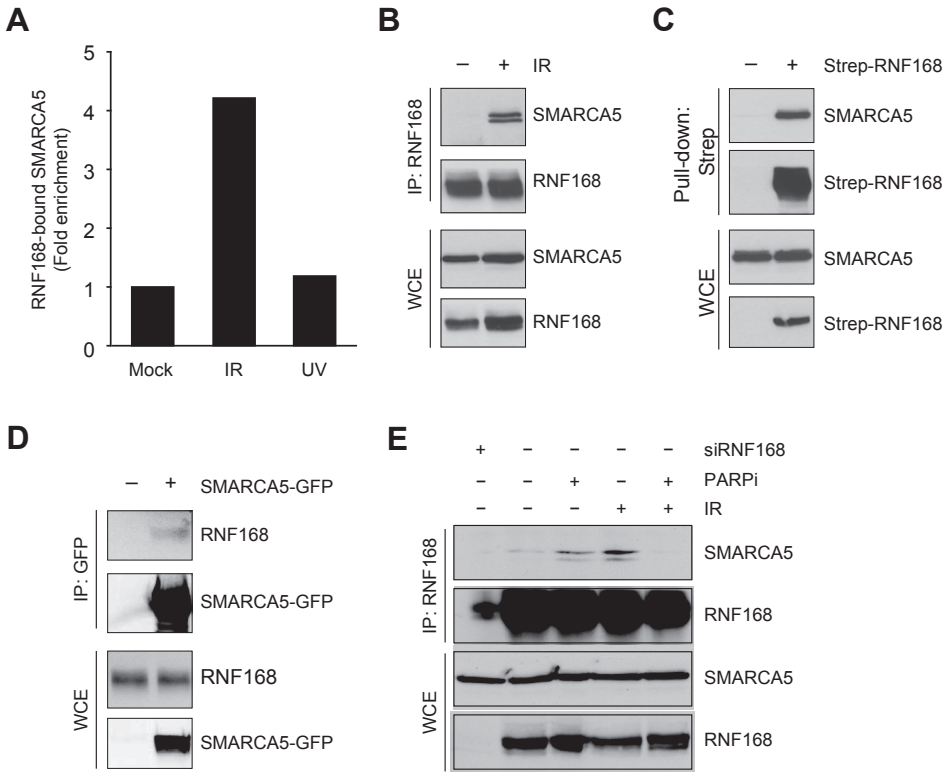


Figure 7. SMARCA5 promotes γ H2AX ubiquitylation and BRCA1 accumulation at DSBs. (A) Cells were transfected with the indicated siRNAs, exposed to 1 Gy IR and 1 h later immunostained for conjugated ubiquitin (FK2) to visualize IRIF. Scale bar, 10 μ m. (B) Quantitative representation of FK2 IRIF formation in A. The average number of foci/nucleus $\pm$ s.e.m. is presented. More than 150 nuclei were scored per time point in independent experiments. *, $P < 0.05$, compared with siLuc (control). (C) Cells were transfected with the indicated siRNAs and exposed to 10 Gy IR. 1 h later chromatin-enriched extracts (CEE) were prepared. CEE were subjected to IP with γ H2AX antibody. Western blot analysis of IP with γ H2AX antibody, and CEE with SMARCA5 antibody is shown. Ponceau S staining is a loading control. (D) Graphical representation of relative γ H2AX ubiquitylation levels determined using results from H. Ratios of unmodified γ H2AX and either mono-ubiquitylated or di-ubiquitylated were calculated and normalized to that of control cells, which was set to 1. The mean $\pm$ s.e.m. of two independent experiments is shown. (E) As in A, except that cells were immunostained for BRCA1. (F) As in B, except that cells from E were analyzed.

we found that SMARCA5 knockdown decreased the levels of IR-induced γ H2AX mono- and di-ubiquitylation, an effect that was similar to that observed after RNF8 knockdown (Fig. 7, c and d) (Huen et al., 2007). Finally, we analyzed the recruitment of the RAP80-BRCA1 complex, which requires direct binding to conjugated ubiquitin for its accumulation at DSBs (Sobhian et al., 2007; Wu et al., 2009). Consistent with the observed defects in ubiquitin conjugation, we found that BRCA1 IRIF formation was impaired in SMARCA5-depleted cells (Fig. 7, e and f) (Nakamura et al., 2011). Together, our findings show that SMARCA5 binds to RNF168 and regulates its assembly at DSBs. Moreover, SMARCA5 is required for the RNF168-induced conjugation of ubiquitin chains and the subsequent recruitment of the ubiquitin-binding BRCA1 complex. These results demonstrate that SMARCA5 regulates the DSB response at the level of RNF168.

DISCUSSION

Recent papers have implicated SMARCA5 in the DSB response (Lan et al., 2010; Nakamura et al., 2011). In line with this work, our current study revealed that SMARCA5 protects cells against IR and regulates the two major pathways of DSB repair, HR and NHEJ. Here, we also uncover an unrecognized role of SMARCA5 in regulating the RNF168 signaling cascade at DSBs. SMARCA5 and RNF168 interact in the DSB-flanking chromatin compartment in a PARP-dependent manner to regulate RNF168-dependent histone ubiquitylation and subsequent BRCA1 assembly. Thus, our findings unveil a novel physical and functional link between DNA damage-induced PARylation, chromatin remodeling and the RNF8/RNF168-signaling cascade (Fig. 8).

SMARCA5 protects against DNA damage

ISW-1 was identified through a genome-wide genetic screen in *C.elegans* as a factor that protects worms against IR (van Haaften et al., 2006). We provide evidence that one of its human orthologue, SMARCA5, also protects cells against the genotoxic effects of IR, indicating a conserved function for these factors in the cellular response to IR. Previous reports not only corroborate these findings, but also showed that SMARCA5 protects cells against the cytotoxic effects of the DSB-inducing agent bleomycin, the DNA alkylating agent methylmethanesulphonate (MMS) and the DNA topoisomerase 1 inhibitor camptothecin (CPT) (Lan et al., 2010). Since these agents induce various types of DNA lesions that trigger different signaling and repair mechanisms, the data suggest that SMARCA5 may play a broad role in the DDR.

The hypersensitivity to IR may result from defects in the signaling and/or repair of DSBs. This could indirectly stem from altered gene expression in response to IR. However, we found that the loss of SMARCA5 did not affect the steady-state levels of key DDR factors (MDC1, RNF8, RNF168, 53BP1, BRCA1, BRCA2, RAD51 and p53), neither before nor after exposure of cells to IR (Fig. S5, a and b). Although a more detailed analysis of the role of SMARCA5 in the transcription of DNA damage-regulated genes may be required, the result thus far suggest that SMARCA5 protects

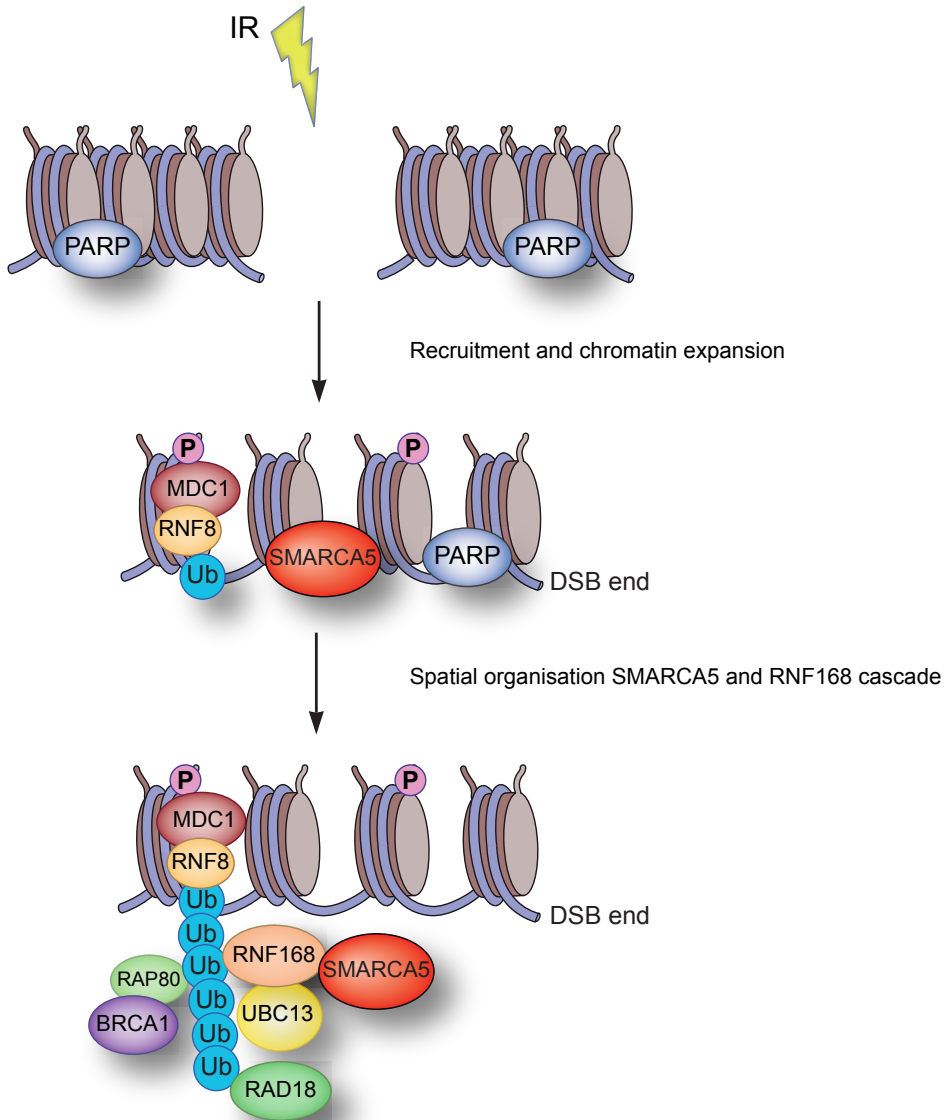


Figure 8. Model for the role of SMARCA5 in the DSB response. A DSB in chromatin triggers PARP recruitment and γ H2AX formation. PARP elicits local chromatin expansion and SMARCA5 recruitment, whereas γ H2AX promotes the accumulation of MDC1 and RNF8, which ubiquitylates H2A-type histones. This leads to the recruitment of RNF168, which amplifies RNF8-induced ubiquitylation to promote the accumulation of ubiquitin-binding factors, including RAD18 and the RAP80-BRCA1 complex. PARP promotes the spatial distribution of SMARCA5 and factors of the RNF168 signaling cascade (RNF168, UBC13, RAD18, RAP80, BRCA1), and stimulates the interaction between SMARCA5 and RNF168 throughout DSB-flanking chromatin, thereby regulating RNF168-dependent signaling of DSBs. SMARCA5 also promotes efficient repair of DSB by NHEJ and HR (see text for details).

human cells against IR by acting directly at DSBs to promote DSB repair. Accordingly, the current work, in agreement with previous reports, shows that SMARCA5 is recruited to DSBs where it regulates DSB repair by both HR and NHEJ (Lan et al., 2010; Nakamura et al., 2011).

SMARCA5: a DSB repair factor and novel target for cancer therapy

How does SMARCA5 affect these major pathways of DSB repair? Loss of SMARCA5 impairs the loading of key factors involved in HR, including RPA, RAD51 and BRCA1, at DSB sites, providing a potential mechanistic basis for the regulation of HR by this chromatin remodeling enzyme (Fig. 1) (Nakamura et al., 2011). Less clear is how SMARCA5 affects DSB repair by NHEJ. SMARCA5 together with ACF1, CHRAC15 and CHRAC17 constitutes the CHRAC complex. ACF1 in this complex interacts with the KU70/KU80 heterodimer and the interaction is enhanced by DNA damage (Lan et al., 2010). Importantly, ACF1, but not SMARCA5 knockdown significantly decreased the accumulation of KU70/KU80 at sites of laser-induced DNA damage (Lan et al., 2010). These results may provide an explanation for the role of ACF1 in NHEJ, but do not reveal how SMARCA5 stimulates CHRAC-driven chromatin remodeling to regulate NHEJ. Further analysis of CHRAC and other SMARCA5-containing complexes will be required to unravel SMARCA5's contribution to NHEJ.

The involvement of SMARCA5 in HR may have consequences for cancer therapeutic strategies that are based on the utilization of PARP inhibitors. HR-defective BRCA1- and BRCA2-deficient cells are highly sensitive to PARP inhibition, which impairs base excision repair and leads to the accumulation of single-strand breaks. It is thought that during DNA replication these breaks are converted into DSBs that are lethal for the cells as they cannot be repaired by BRCA1/2-dependent HR (Farmer et al., 2005; McCabe et al., 2006). We found that SMARCA5-depleted cells were also extremely sensitive to PARP inhibition (Fig. 1 e), consistent with a role for SMARCA5 in HR. These findings suggest that PARP inhibition might not only be a useful therapeutic strategy for the treatment of BRCA1- or BRCA2-deficient tumors, but also for the treatment of SMARCA5-deficient tumors displaying deficiencies in HR (Cetin et al., 2008).

PARP and the recruitment of SMARCA5 to DSBs

SMARCA5 is recruited to both site-specific chromosomal DSBs induced by the *I-SceI* or *AsiSI* nucleases and to laser-induced DNA damage (Fig. 1 f; Fig. S3, c and d) (Lan et al., 2010; Nakamura et al., 2011; Erdel et al., 2010; Sanchez-Molina et al., 2011). Nakamura and co-workers examined how SMARCA5 is recruited to DNA breaks and implicated a role for the RNF20 E3 ubiquitin ligase in this process (Nakamura et al., 2011). This ligase promotes mono-ubiquitylation of histone H2B, which in turn triggers methylation of histone H3 at lysine 4 (H3K4me) in DSB-flanking chromatin. It is thought that SMARCA5 binds to the latter chromatin mark within damaged chromatin compartments (Nakamura et al., 2011; Santos-Rosa et al., 2003). In contrast, another report suggested a role for ACF1 in DSB recruitment of SMARCA5, although formerly

it was not shown that loss of ACF1 abrogates SMARCA5 assembly at DSBs (Lan et al., 2010). In the current work we extend these findings by demonstrating that SMARCA5 is rapidly recruited to DSBs in a manner that requires the activity of PARP enzymes, but does not involve ATM, DNA-PK, MDC1 or RNF168 (Fig. 2). Given that RNF20 and potentially ACF1 also contribute to SMARCA5 assembly at DNA strand breaks (Lan et al., 2010; Nakamura et al., 2011), it will be of interest to unravel how the crosstalk between PARP activity, RNF20-mediated H2B mono-ubiquitylation and ACF1 orchestrates SMARCA5 accumulation and/or activity at DSB sites.

How does PARP activity affect SMARCA5 accumulation at DSB sites? It is known that PARP not only targets itself for PARylation, but also several other non-histone and histone proteins involved in the DSB response (Messner and Hottiger, 2011). These PARylated proteins may play an important role in the recruitment of DDR factors that display PAR binding activity. Two chromatin remodeling enzymes, ALC1 and CHD4, contain regions that display PAR binding and are required for efficient recruitment of these enzymes to sites of DNA damage (Polo et al., 2010; Gottschalk et al., 2009; Chou et al., 2010; Ahel et al., 2009). SMARCA5 does not contain a canonical macro-domain or PBZ zinc finger domain for PAR binding. It harbors a putative PAR-binding sequence, but a recent report demonstrated that this sequence was dispensable for PAR binding *in vitro* (Gagne et al., 2008), suggesting that it may not play a major role in the accumulation of SMARCA5 at DNA strand breaks. In line with this, mutation analysis of the identified putative PAR binding sequence suggested that it is also dispensable for the PARP-dependent accumulation and spreading of SMARCA5 in DSB-tracks (Fig. S6). This suggests that other regions in SMARCA5 may be responsible for the association with PARylated proteins and spreading within the damaged chromatin compartment. Alternatively, one of the many binding partners of SMARCA5 may contain a PAR-binding domain that contributes to SMARCA5 accumulation at sites of DNA damage and, together with SMARCA5, may play an important role in the signaling and repair of DSBs. Finally, SMARCA5 may be PARylated after DNA damage (Gagne et al., 2008). As such we cannot exclude the possibility that this post-translational modification affects SMARCA5 function during the DSB response.

PARP links SMARCA5 to the RNF168 signaling cascade at DSBs

The fact that PARP1 is rapidly recruited to DNA strand breaks is in line with its role in the early recruitment of SMARCA5 to DSBs (Haince et al., 2008). Given that PARP contributes to SMARCA5 recruitment and SMARCA5 regulates efficient RNF168 and BRCA1 assembly at DSBs, our work suggests that PARP modulates RNF168-dependent ubiquitylation that elicits BRCA1 accumulation at DNA breaks.

We showed that PARP inhibition moderately affects chromatin expansion at sites of laser-induced DNA damage (Fig. 3; Fig. S4 b), which is in line with previous work suggesting that laser micro-irradiation evokes chromatin expansion independently of ATM or γ H2AX (Kruhlak et al., 2006). Conversely, we found PARP inhibition to selectively impair the spreading of SMARCA5, as well as that of RNF168 and several

of its downstream factors into DSB-flanking chromatin regions (Fig. 3; Fig. S4 b). This suggests that PARP affects the distribution or the spreading of a specific subset of RNF168-associated factors into damaged chromatin regions, while it is dispensable for the spreading of other factors, such as RNF8 and MDC1. Given that SMARCA5 is recruited to DSBs in a partially PARP-dependent manner and considering that SMARCA5 interacts with RNF168 in damaged chromatin in a PARP-dependent manner, it is tempting to speculate that SMARCA5 is involved in regulating the spreading of repair and signaling factors involved in the RNF168 response. However, whether this involves the binding of signaling factors to PAR chains, or the local chromatin remodeling activity of SMARCA5 is currently unclear. Moreover, we can also not rule out the possibility that this mechanism involves the PARylation of signaling factors or chromatin components.

But what is the functional relevance of the role of PARP in the distribution of SMARCA5 and different DDR factors? One possibility is that the correct distribution of these factors is important for their sustained retention at DSBs. Such a scenario would imply that SMARCA5 is required for the accumulation of these factors at DSBs. In support of this we found that SMARCA5 loss affects the accumulation of RNF168, ubiquitin and BRCA1, but not that of MDC1 and RNF8, at DSBs (Fig. 5, 6 and 7). Moreover, we demonstrated that SMARCA5 physically interacts with RNF168 in a PARP-dependent manner in the DSB-flanking chromatin compartment (Fig. 4). Thus, our work demonstrates that the interplay between PARP activity, SMARCA5 chromatin remodeling and the RNF168 cascade plays an important role in the DSB response. We propose a model, in which SMARCA5 and RNF168 are recruited to DSBs through pathways that involve PARP and ATM/ γ H2AX/MDC1/RNF8, respectively (Fig. 8) (Doil et al., 2009; Stewart et al., 2009). Once recruited, PARP activity not only promotes the redistribution of SMARCA5 and factors of the RNF168 cascade, but also stimulates the physical interaction between SMARCA5 and RNF168, which, in turn, promotes ubiquitin conjugation, and the subsequent accumulation of BRCA1 in DSB-flanking chromatin (Fig. 8).

SMARCA5 and other chromatin remodelers involved in the RNF168 cascade

We recently reported that CHD4 also affects BRCA1 accumulation by promoting the ubiquitin signaling cascade at the level of RNF8/RNF168 (Larsen et al., 2010; Smeenk et al., 2010). Since Polo and colleagues demonstrated that CHD4 is recruited to DSB-containing tracks in a PARP-dependent manner (Polo et al., 2010), it will be important to examine whether PARP not only modulates SMARCA5-, but also CHD4-dependent loading of RNF168 and BRCA1 at DSBs. Additionally, p400, which is a SNF2 type ATPase that forms the catalytic subunit of the NuA4 complex, is yet another chromatin remodeling factor that is required for the RNF8/RNF168-dependent ubiquitination of chromatin, and the subsequent accumulation of BRCA1 at DSBs (Doyon and Cote, 2004; Xu et al., 2010). For both p400 and CHD4, it remains unclear how their chromatin

remodeling activity regulates RNF8/RNF168-dependent signaling of DSBs. Based on our finding that SMARCA5 affects the ubiquitin cascade at DSBs by interacting with the RNF168 ubiquitin E3 ligases, it will be of interest to study whether CHD4 and/or p400 interact with RNF8, RNF168 or other DDR components involved in the RNF8/RNF168 signaling cascade. We speculate that such interactions may be important to induce local chromatin changes that are required for RNF8/RNF168-mediated histone ubiquitylation and the subsequent accumulation of BRCA1 at DSBs. To address this, one would need to establish how CHD4, p400 and SMARCA5 remodel chromatin in the vicinity of DSBs. The findings presented here thus far reveal that the interplay between DNA damage-induced PARylation, SMARCA5-mediated chromatin remodeling and the RNF8/RNF168 ubiquitin cascade regulates the cellular response to DSBs and as such promotes genomic stability in response to genetic insult.

MATERIALS AND METHODS

Cell culture and chemicals

U2OS, HEK293, HeLa, VH10-SV40, VH10-hTERT and XP2OS-SV immortalized fibroblasts were grown in DMEM (Gibco) containing 10% FCS (Bodinco BV). XPA-GFP was expressed in XP2OS-SV cells, which are deficient in XPA (Rademakers et al., 2003). U2OS cells stably expressing GFP-MDC1, GFP-RNF168 or GFP-53BP1 were gifts from Jiri Lukas. GFP-RAP80 and GFP-RAD18 expression vectors were obtained from Anton Jetten and Satoshi Tateishi, respectively. The cDNA for human SMARCA5 (Open Biosystems) was cloned into pEGFP-N1 (Clontech). SMARCA5-GFP was stably expressed in U2OS cells. siRNA and plasmid transfections were performed using HiPerfect (Qiagen) and JetPEI (Polyplus Transfection), respectively, according to the manufacturer's instructions. The following siRNA sequences were used: 5'-CGUACGCGGAUACUUCGA-3' (Luciferase), 5'-GGAUUAACUGGCUCAUUU-3' (SMARCA5-1, Dharmacon), 5'-GAGGAGAUGUAAUACCUUAUU-3' (SMARCA5-2, Dharmacon), 5'-GGAAUGGUUACUCGGAUA-3' (SMARCA5-5, Dharmacon), 5'-GGGCAAUAGAUUCGAGUA-3' (SMARCA5-6, Dharmacon); 5'-GAGGGCCAAUGGACAAUUA-3' (RNF8, Dharmacon); 5'-AUAUGUUGGUGAACUGAGA-3' (XRCC4) (Sartori et al., 2007), 5'-GAAGAAUGCAGGUUUAUA-3' (BRCA2, MWG), 5'-CAGAAGAGUGCCACUGUA-3' (MDC1, MWG). siRNAs against RNF168 were used as described (Doil et al., 2009). Cells were examined 48 h after siRNA transfection unless otherwise stated. DNA-PK inhibitor (NU7026, EMD Biosciences) was used at a concentration of 10 μ M. ATM inhibitor (KU-55933) and PARP inhibitor (KU-0058948) were gifts from Mark O'Connor and used at a concentration of 10 μ M.

Generation of DSBs

DSBs were induced by IR, which was delivered by the YXlon X-ray generator (YXlon International, 200 KV, 4 mA, dose rate 1.1 Gy/min).

Cell survival assay

VH10-SV40 cells were transfected for 48 h, trypsinized, seeded at low density and exposed to IR or PARP inhibitor. 7 days later cells were washed with 0.9% NaCl and stained with methylene blue. Colonies of more than 20 cells were scored.

Microscopy and laser micro-irradiation

Brightfield pictures were taken with an EVOS fl fluorescence microscope (AMG, Westover Scientific) using the 4x Ph objective. Laser micro-irradiation was carried out on a Leica SP5 confocal microscope equipped with an environmental chamber set to 37°C and 5% CO₂. Cells were grown on glass cover slips. DSB-containing tracks (1.5 µm width) were generated with a Mira modelocked Ti:Sapphire laser (λ = 800 nm, pulselength = 200 fs, repetition rate = 76 MHz, output power on the cells for DNA damage induction = 80 mW). Confocal images were recorded before and after laser irradiation at 5 sec time intervals over a period of 5 min and analyzed using LAS-AF software. Fluorescence intensities were subtracted by the pre-bleach values and normalized to the first datapoint, which was set 0, to obtain Relative Fluorescence Units (RFU). Track width was measured using ImageJ software.

Antibodies

IRIF and western blot analysis were performed using antibodies to γ H2AX, phospho histone H3 S10p (Millipore), α -Tubulin (Sigma), Strep (IBA BioTAGnology), GFP (Roche), ubiquitin (FK2, Enzo Life Sciences), BRCA1 (Calbiochem and Santa Cruz), SMARCA5/SNF2h, histone H3 and MDC1 (Abcam). The antibodies to RNF168, RAD51 and XRCC4 were gifts from Daniel Durocher, Roland Kanaar and Mauro Modesti, respectively.

IRIF analysis

IRIF were analyzed as described previously (Smeenk et al., 2010). Briefly, cells were grown on glass cover slips and treated as indicated in the figure legends. Subsequently, cells were washed with PBS, fixed with 2% formaldehyde and 0.25% Triton X-100 in PBS and incubated with appropriate primary and secondary antibodies (AlexaFluor 488, Invitrogen or goat anti-rabbit IgG conjugated to Cy3, Jackson Immunoresearch) and DAPI. An additional extraction with 0.25% Triton in CSK buffer (10 mM Hepes, pH 7.4, 300 mM sucrose, 100 mM NaCl, 3 mM MgCl₂) was performed prior to fixation and incubation with primary antibodies against RNF168 and BRCA1. Images were recorded with a Zeiss Axioplan microscope using a 63x/1.25 NA oil objective and an Axiocam mRm camera. IRIF were analyzed using home-made Stacks software as described (Smeenk et al., 2010). Statistical significance was established at each timepoint using one-way ANOVA.

Chromatin fractionation, immunoprecipitation and western blotting

Chromatin-enriched extracts were prepared and used for immunoprecipitation of γ H2AX as described previously (Huen et al., 2007; Smeenk et al., 2010). Whole cell

extracts (WCE) were prepared by cell lysis in Laemmli buffer. Proteins were separated in Bis-Tris-HCl-buffered acrylamide gels (Invitrogen) and blotted onto PVDF membrane (Millipore). Membranes were incubated with primary antibody as indicated in the figure legends, followed by incubation with secondary antibody (Odyssey IRDye® Li-Cor Biosciences). The Odyssey imager (Li-Cor Biosciences) equipped with Li-Cor Odyssey 3.0 software was used to scan the membranes and analyze the fluorescence signals. For the RNF168-SMARCA5 interaction studies cells were lysed in EBC buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% NP-40, 1 mM EDTA) supplemented with protease and phosphatase inhibitor cocktails. The cleared lysates were subjected to immunoprecipitation with GFP Trap beads (Chromotek), Strep-Tactin sepharose (IBA BioTAGnology), or RNF168 antibody for 1.5 h, and the beads were then washed 4 times with EBC buffer and boiled in sample buffer. Bound proteins were resolved by SDS-PAGE and processed for immunoblotting. For mass spectrometric analysis of RNF168-SMARCA5 interaction, RNF168 immunoprecipitates from cells subjected to SILAC labeling for 7 days were eluted from beads with 0.1 M glycine (pH 2.5) and analyzed on an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) (Ong et al., 2002). Data analysis was performed with MaxQuant software.

Homologous Recombination and Non-Homologous End-Joining assays

HEK293 cell lines containing either a stably integrated copy of the DR-GFP or EJ5-GFP reporter were used to measure the repair of I-SceI-induced DSBs by HR or NHEJ, respectively (Pierce et al., 1999; Bennardo et al., 2008). Briefly, 48 h after siRNA transfection, cells were transfected with the I-SceI expression vector pCBASce and a RFP expression vector (Pierce et al., 1999). 48 h later the fraction of GFP-positive cells among the RFP-positive cells was determined by FACS on a BD LSRII flow cytometer (BD Bioscience) using FACSDiva software version 5.0.3. Quantifications were performed using WinMDI 2.9 software.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY FIGURES

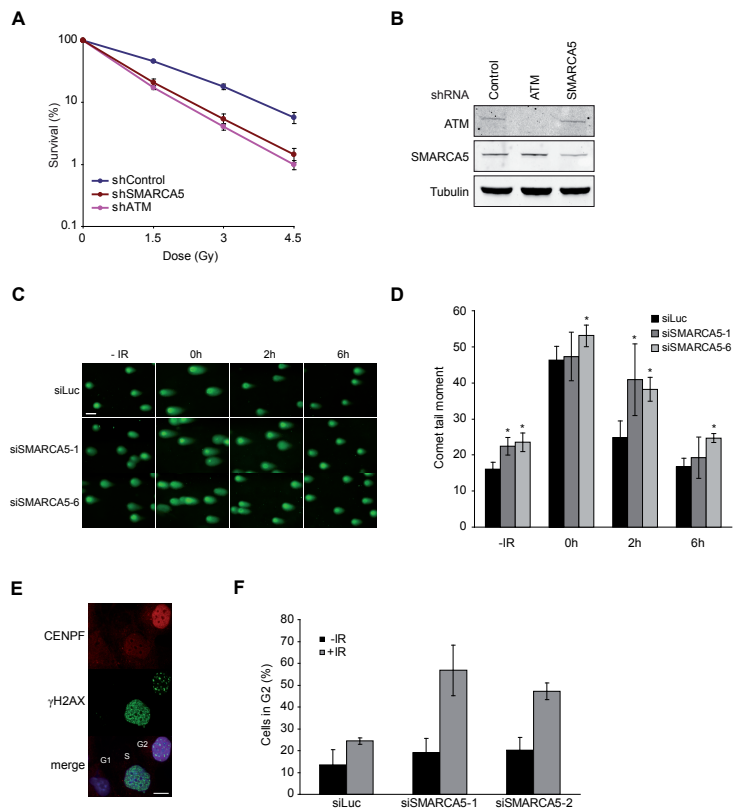


Figure S1. SMARCA5 depletion renders cells hypersensitive to IR and impairs DSB repair and cell cycle progression after IR. (A) VH10-hTERT cells stably expressing an shRNA against SMARCA5 (5'-gatccccGTGTTTGCTTCAAAGGAAA ttaagagaTTTCCTTTGAAGCAAACACTtttt-3') or ATM displayed increased IR sensitivity when compared to control cells stably expressing mouse HIF1 shRNA in clonogenic survival assays (van Haaften et al., 2006). Graphs represent the mean $\pm$ s.e.m. of 3 independent experiments. (B) SMARCA5 levels were monitored by western blot analysis using WCE of cells in A. Tubulin is a loading control. (C) VH10-hTERT cells were transfected with the indicated siRNAs for 72 h, exposed to 20 Gy IR and subjected to neutral comet analysis at the indicated time points using the Comet Assay system (Trevigen) according to the manufacturer's instructions. Representative images are shown. Scale bar, 30 μ m. (D) Quantification of tail moments using cells from C. Comet tail moments were scored using Comet Score software (TriTek). Tail moments for each condition were calculated for at least 100 cells per data point. The mean $\pm$ s.e.m. of 3 experiments is shown. Statistical significance was established at each timepoint using a student t-test. *, $P < 0.05$, compared with siLuc (control). (E) Knockdown of SMARCA5 induces a G2 cell cycle arrest after exposure of cells to IR. U2OS cells were exposed to 5 Gy IR or left untreated and after 18 h immunostained for γ H2AX and CENP-F (using anti-CENP-F antibody; Santa Cruz). DNA was stained with DAPI. The levels of CENP-F staining distinguished G1, S and G2 cells. (F) The percentage of G2 cells $\pm$ s.e.m. is presented. More than 120 nuclei were scored per sample in at least 2 independent experiments.

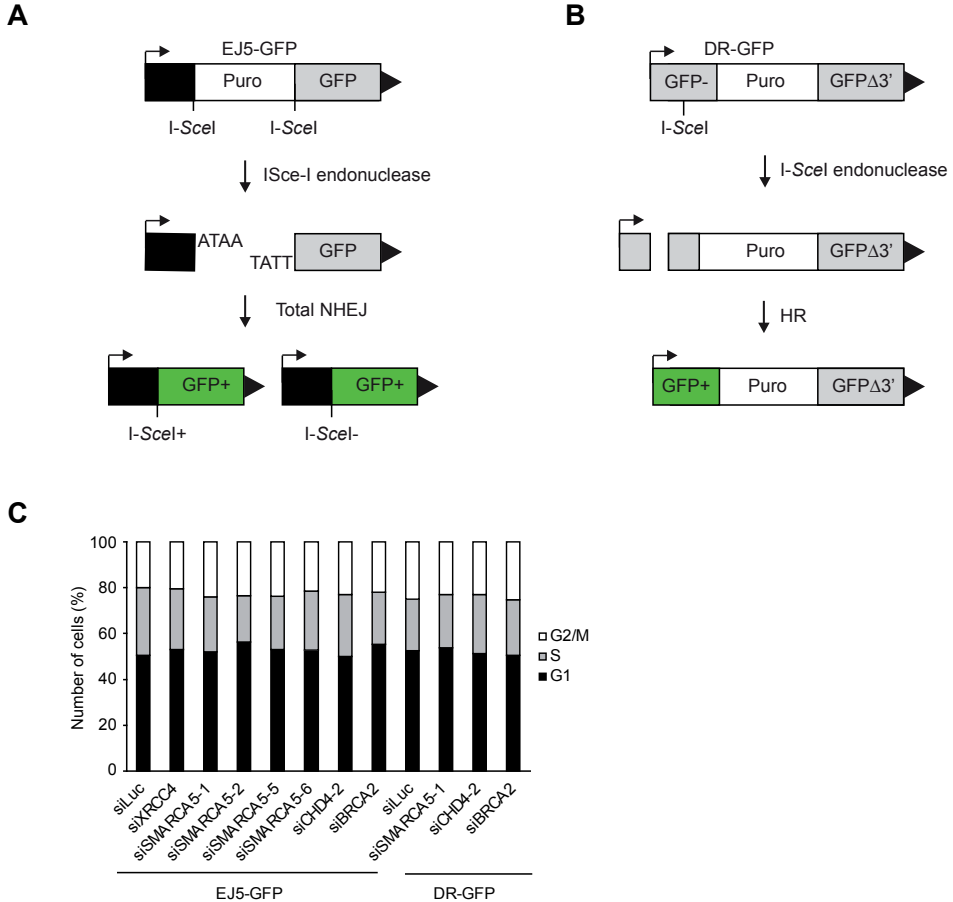


Figure S2. SMARCA5 depletion does not affect cell cycle progression in NHEJ and HR reporter cell systems. (A) Schematic representation of a reporter system for NHEJ. EJ5-GFP consists of a promoter that is separated from a GFP cassette by insertion of a puromycin resistance marker flanked by I-SceI recognition sites (Bennardo et al., 2008). After transient expression of I-SceI and subsequent cleavage at these sites, NHEJ (either error-free or error-prone) will fuse the promoter to GFP and restore expression. (B) Schematic representation of a reporter system for HR. DR-GFP consists of two GFP alleles that are non-functional due to the insertion of an I-SceI recognition site and a 3' truncation, respectively (Weinstock et al., 2006). After transient expression of I-SceI and subsequent cleavage at the I-SceI recognition site, HR will use the GFPΔ3' as a template to repair the DSB, which will restore GFP expression. (C) HEK293T cells containing the EJ5-GFP or DR-GFP reporter system were transfected with the indicated siRNAs. After 48 h cells were stained with propidium iodide and subjected to FACS. Percentage of cells in G1 (black bar), S (grey bar) and G2/M (white bar) phase is represented. Data shown are the average of 2 independent experiments.

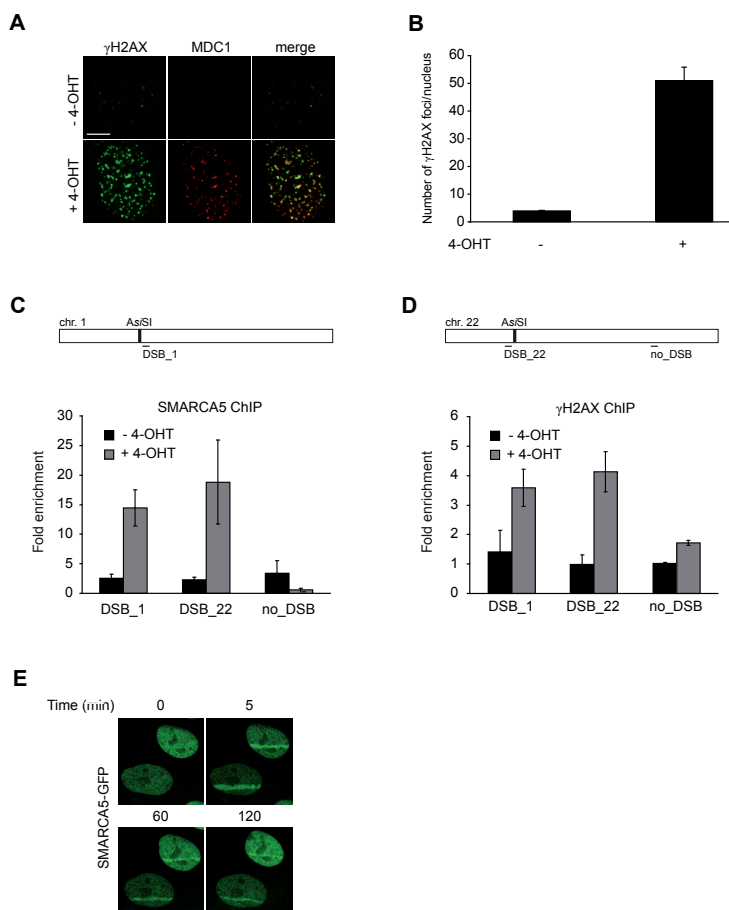


Figure S3. SMARCA5 accumulates at site-specific DSBs and laser-induced DNA damage.

(A) U2OS cells containing AsiSI-ER display increased 4-OHT-induced γ H2AX and MDC1 foci. Cells containing AsiSI-ER were untreated or treated with 300 nM 4-OHT for 4 h and subsequently immunostained for γ H2AX and MDC1. (B) Quantitative analysis of γ H2AX focus formation. The average number of foci/nucleus $\pm$ s.e.m. is presented. More than 150 nuclei from cells in A were scored per time point in at least 2 independent experiments. (C) Site-specific DSBs were induced by treatment of U2OS cells containing AsiSI-ER with 300 nM 4-hydroxytamoxifen (4-OHT) for 4 hours. Cells were analyzed by ChIP using antibodies against SMARCA5, followed by qPCR using primers at the indicated distances from AsiSI consensus sequences on chromosome 1 (position 89.231.183) and chromosome 22 (position 19.180.307) as described previously (Iacovoni et al., 2010). The following qPCR primers were used: DSB_1 forward 5'-GATTGGCTATGGGTGTGGAC-3' and reverse 5'-CATCCTTGCAAACAGTCT-3'; DSB_22 forward 5'-CCTTCTTCCCAGTGGTTCA-3' and reverse 5'-GTGGTCTGACCCAGAGTGGT-3'; no_DSB forward 5'-CCCATCTCAACTCCACACT-3' and reverse 5'-CTTGTCAGATTCGCTGTGA-3'; GAPDH forward 5'-GAAGGTGAAGGTCGGCGTCA-3' and 5'-GAAGATGGTGATGGGATTTC-3'. Values for the cleaved (DSB_1 and DSB_22) and non-cleaved (no_DSB; 2 Mb distal to DSB_22) sites were normalized to those for the GAPDH control in ChIP and input samples. ChIP ratios were normalized to input ratios. (D) As in C, except that an antibody against γ H2AX was used. (E) U2OS cells expressing SMARCA5-GFP were laser-irradiated and protein assembly at the damaged area was monitored at the indicated timepoints. Scale bars, 10 μ m.

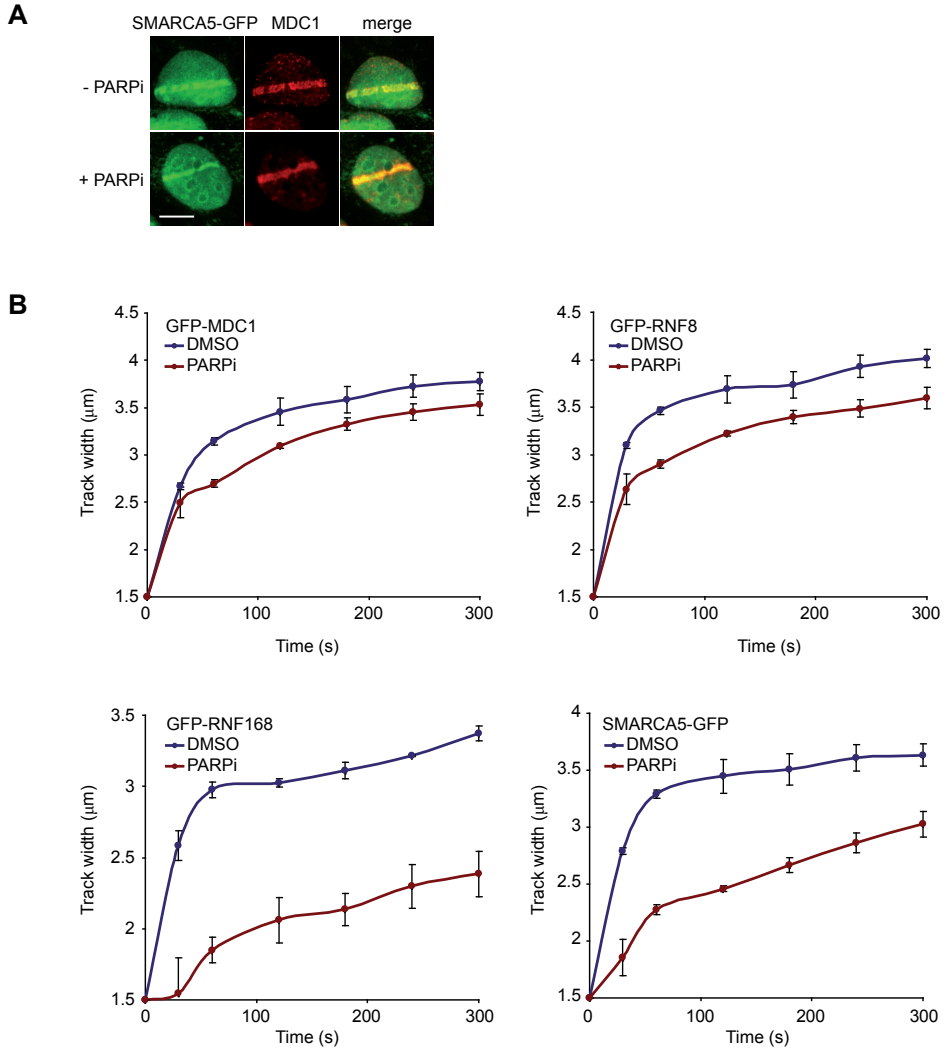
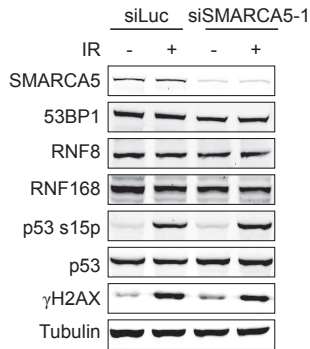


Figure S4. PARP affects the occupancy of SMARCA5 and factors of the RNF168 cascade throughout damaged chromatin. (A) U2OS cells expressing SMARCA5-GFP were treated with PARP inhibitor or left untreated, then subjected to multi-photon laser-irradiation and after 180 seconds analyzed for the expansion of SMARCA5-GFP and immunostained endogenous MDC1. Representative images illustrating the effect of PARP inhibition on the expansion of SMARCA5-GFP and MDC1 are shown. Scale bars, 10 μm. (B) As in A, except that cells expressing GFP-MDC1, GFP-RNF8 or GFP-RNF168 were included. Tracks with a width of 1.5 μm were induced using a multiphoton laser and the subsequent increase in track width was measured up to 300 seconds after irradiation. The average track width $\pm$ s.e.m. for at least 20 tracks from two independent experiments is presented.

A



B

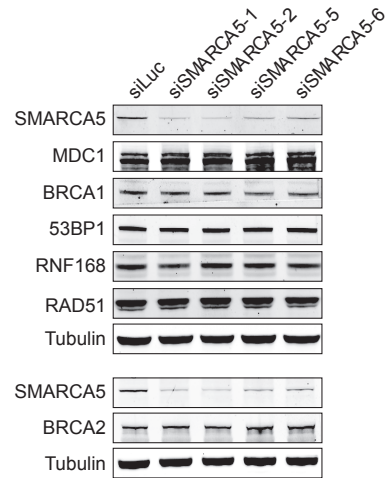


Figure S5. SMARCA5 depletion does not affect the expression levels of DDR proteins. (A) SMARCA5 knockdown does not affect the expression levels of several DDR proteins. U2OS cells were transfected with the indicated siRNAs and left untreated or irradiated with 10 Gy IR. 1 h later WCE were prepared and protein levels were monitored by western blot analysis. Anti-53BP1 (Novus Biologicals), anti-p53 (Santa Cruz), anti-p53 S15p (Cell Signaling), anti-RNF8 (Abcam) and anti-BRCA2 (AB-1, Oncogene) antibodies were used. (B) As in A, except that cells were not irradiated.

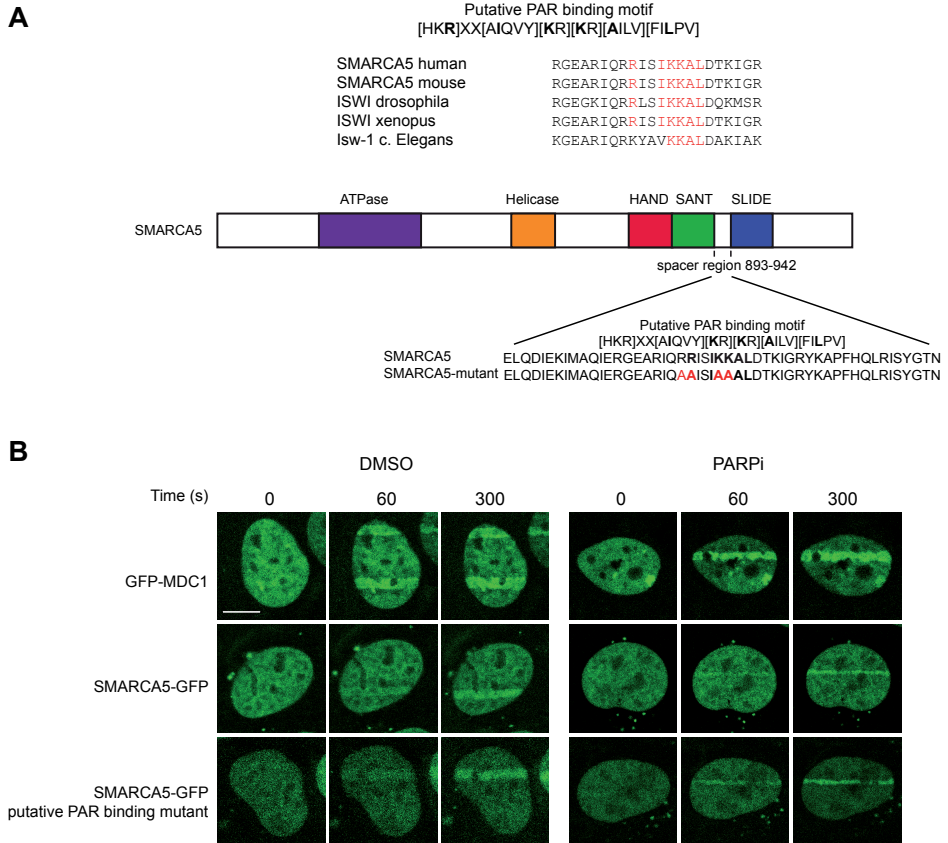


Figure S6. The putative PAR binding domain of SMARCA5 is not required for its accumulation and spreading in damaged chromatin (A) Schematic representation of the different functional domains in the SMARCA5 protein. A highly conserved putative PAR binding motif was found in the spacer region (893-943) located between the SANT and SLIDE domains. A SMARCA5 mutant was generated in which four conserved residues (two arginine residues and two lysine residues) in the putative PAR binding motif were substituted for alanine residues (indicated in red). (B) U2OS cells expressing GFP-MDC1, SMARCA5-GFP or the SMARCA5-GFP putative PAR binding mutant were treated with PARP inhibitor or left untreated and then subjected to multi-photon laser-irradiation.

