Molecular and Cellular Pathobiology

Cancer Research

Germline Mutations in BAP1 Impair Its Function in DNA Double-Strand Break Repair

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Abstract

The BRCA1-associated deubiquitylase BAP1 is mutated in several cancers, most notably mesothelioma and melanoma, where it is thought to promote oncogenesis. In this study, we present evidence that BAP1 functions as part of the DNA damage response (DDR). We found that BAP1 mediates rapid poly(ADP-ribose)-dependent recruitment of the polycomb deubiquitylase complex PR-DUB to sites of DNA damage. Furthermore, we identified BAP1 as a phosphorylation target for the DDR kinase ATM. Functionally, BAP1 promoted repair of DNA double-strand breaks, enhancing cell survival after DNA damage. Our results highlight the importance of ubiquitin turnover at sites of DNA damage, and they provide a mechanism to account for the tumor-suppressive function of BAP1. *Cancer Res; 74(16); 4282–94. ©2014 AACR.*

Introduction

Posttranslational modification of proteins with ubiquitin plays a central role in the DNA damage response (DDR; refs. 1 and 2). Ubiquitylation is functionally linked to transcriptional silencing (3–5), DNA damage signal amplification, and DNA repair (2, 6–14). Recent studies indicate that the ubiquitin landscape at damaged chromatin is very dynamic, and a significant repertoire of deubiquitylating enzymes (DUB) or ubiquitin-specific proteases (USP) are emerging as critical regulators of ubiquitin signaling.

The balance between E3 ubiquitin ligases and DUB activities defines the ubiquitin landscape and ubiquitin dynamics at DNA double-strand breaks (DSB; ref. 15). BRCC36 is the best characterized DUB involved at DSBs. It controls the breakdown of K63-linked polyubiquitin chains (16, 17). There are also several other DUBs that function at DSBs, although many do not accumulate in DSB foci. For example, USP1 deubiquitylates monoubiquitylated FancD2 (18, 19), whereas USP3 is thought to act on ubiquitylated H2A at lysine 119 (Ub-H2AK119; ref. 20). Additional DUBs have been found to interact with proteins found at DSBs, including USP28 (21), which stabilizes 53BP1, and STAMBP and OTUB1, which interact with Ubc13 (22). Given the different substrate specificities, DUB regulation is likely to fine-tune the relative ratios of different ubiquitin linkages at DNA lesions as well as overall ubiquitin density to modulate DNA damage signaling.

BAP1 is a 729 amino acid protein that was originally isolated as a nuclear DUB that interacts with, and enhances the growth-suppressive effect of, the tumor suppressor BRCA1 (23). It was predicted that autoubiquitylated BRCA1 would be a substrate for BAP1 DUB activity (23), but there is no evidence to support this. BAP1 also acts in a BRCA1-independent manner; its overexpression in cells lacking BRCA1 has been shown to inhibit cell proliferation and tumor growth (24). Interestingly, recent studies indicate that RNA interference–mediated depletion of BAP1 can also exert an inhibitory effect on cell proliferation (25–27). Although the exact molecular mechanisms are largely unknown, these data suggest that BAP1 controls cell-cycle progression.

Histone H2A is mono-ubiquitylated at Lys119 (Ub-H2AK119) by the polycomb repressor complex 1 (PRC1) and is critical for transcriptional silencing of the developmentally regulated HOX genes and X chromosome inactivation (3, 28). Recently, the *Drosophila* polycomb group protein Calypso was found to be the ortholog of BAP1. Calypso associates with ASX to form the polycomb repressive deubiquitylase (PR-DUB), which in turn deubiquitylates histone H2A and regulates hox gene expression by antagonizing PRC1 function (29, 30). BAP1 has *in vivo* tumor-suppressive activity that is dependent on its deubiquitylation activity, and nuclear localization (24). This tumor-suppressive function is independent of BRCA1 expression, providing further evidence that BAP1 deubiquitylation activity targets other substrates *in vivo* (24). In support of BAP1 functioning as a tumor suppressor, several recent publications have identified BAP1 mutations in various forms of cancer (31–36). Germline mutations in BAP1 have been shown to predispose patients to uveal melanoma, lung carcinoma, meningioma, and melanocytic tumors (31–36). BAP1 is also

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi: 10.1158/0008-5472.CAN-13-3109

commonly inactivated by somatic mutations and/or its location on chromosome 3p21.1 is lost in malignant pleural mesotheliomas (MPM; ref. 37).

In this study, we describe the participation of BAP1 in the regulation of DSB signaling and repair. This may provide a mechanism for BAP1 to directly contribute to genome stability.

Materials and Methods

Cell transfection, immunoblotting, and immunostaining

U2OS (HTB-96) and H226 cells (CRL-5826) were obtained from the American Type Culture Collection, which authenticated cells via short tandem repeat profiling of each cell line before shipment. The cells were cultured for 2 to 3 weeks to generate stocks that were then kept frozen until used for the current experiments. These cells were cultured in DMEM medium containing 10% fetal calf serum (FCS) at 37°C and 5% CO_2. DR-GFP U2OS and EJ5GFP cells (a gift from Dr. J. Stark) were grown in Mycos 5A media without pyruvate supplemented with 10% FCS. H2AX wild-type (WT) and H2AX knockout mouse embryonic fibroblasts (a gift from André Nussenzweig) were grown in DMEM supplemented with 10% FCS. All deletion mutants were generated by using the QuikChange Site-Directed Mutagenesis Kit (Qiagen) and verified by sequencing. Unless otherwise stated, cells were irradiated in ambient air using a model CS-600 ^{137}Cs irradiator (Picker) at a dose rate of 2 Gy/min. Two different BAP1-specific shRNA were used as described previously (38). We used the following BRCA1 siRNAs: GGAACCUGUCUCCACAAAG-dTdT. KU-55933 (ATM inhibitor), KU-57788 (DNA-Pkcs inhibitor), and Wortmannin were purchase from Selleckchem, dissolved in DMSO, and kept in aliquots at −80°C. Cell transfections with plasmid DNA or siRNA duplexes were performed by using Lipofectamine 3000 and Lipofectamine RNAiMax (Invitrogen), respectively, following the manufacturer's instructions. Immunofluorescene staining and immunoblot analysis were performed as described previously (12). Briefly, cells were permabeabilzed with cytoskeleton (CSK) buffer (100 mmol/L NaCl, 300 mmol/L sucrose, 10 mmol/L pipes, pH 6.8, 3 mmol/L $MgCl_2$, 0.5% Triton X-100) for 2 minutes before being fixed at the indicated time points after exposing cells to IR or laser microirradiation. Cells were fixed with 4.0% paraformaldehyde in PBS, pH 7.5, for 20 minutes at room temperature. Cells were then immunostained as previously described (12). A panel of commercially available primary antibodies γ-H2AX (Millipore), BAP1 (Bethyl), BRCA1 (Santa Cruz Inc.), 53BP1 (Active Motif), BMI1 (Bethyl), MDC1 (Active Motif), RAD51 (Santa Cruz Inc.), ASXL1 (Santa Cruz Inc.), p-ATM-S1981 (Active Motif), CtBP-interacting protein (CtIP; Active Motif), RPA32 (Abcam), and Ub-H2AK119 (Cell Signalling Inc.) were used. The preparation of chromatin fractions was described previously (12). Western blot analysis and chromatin immunoprecipitation (ChIP) experiments were done as previously described (39). BAP1 phosphorylation was detected using phospho-BAP1 (Ser592) antibody (Cell Signalling Inc). PAR polymer blot analysis was conducted to test the ability of BAP1 (Boston Biochem), ASXL1 (Novus), and a combination of BAP1/ASXL1 to bind PAR chains *in vitro* as previously described (40).

Laser microirradiation

We carried out laser microirradiation as a previously described (12). Briefly, cells grown on coverslips were labeled with 1 μg/mL Hoechst 33258 for 10 minutes, and then placed on the stage of a Zeiss LSM510 NLO laser-scanning confocal microscope. DSBs were generated along a 0.2- to 0.8-μm-wide rectangle region across nucleus of a single living cell by excitation of the Hoechst 33258 dye using a near-infrared 780-nm titanium–sapphire laser line. The laser output was set to 10% (unless stated otherwise), and we used 10 iterations to generate localized DSB clearly visible with a ×40 objective. Protein accumulation within the laser path was compared with an undamaged region within the same microirradiated cell. Therefore, each cell provides a reference-unirradiated region that provides high sensitivity to detect and measure small changes in the concentration of fluorescently tagged proteins. For microirradiation, we selected cells with low expression levels and normalized the fluorescence intensity in the microirradiated area to the initial fluorescence in the whole nucleus in order to compensate for photobleaching during acquisition. The average accumulation ± SE of fluorescently tagged proteins from at least 15 cells from two to three independent experiments was plotted. Fluorescence recovery after photobleaching (FRAP) experiments were conducted essentially as described (12).

DNA repair and cell survival assays

Homologous recombination (HR) and nonhomologous end-joining (NHEJ) assays were performed as described previously using the DR-U2OS cell line (DR-GFP) or EJ5GFP cells (41). Cell survival was performed as described previously (12).

Results

BAP1 is recruited to sites of DNA damage

To explore the involvement of BAP1 in the DDR, we first examined whether or not it was recruited to DNA lesions. Laser microirradiation was utilized to generate localized DNA damage in human U2OS cells. The DNA damage sites were visualized by immunofluorescence (IF) microscopy using antibodies to phosphorylated histone H2AX (γ-H2AX). Using an antibody to BAP1, we observed BAP1 accumulation in the damaged regions that colocalized with γ-H2AX tracks (Fig. 1A). Knockdown of BAP1 leads to loss of BAP1 IF signal, demonstrating the specificity of the antibody (Supplementary Fig. S1A). Notably, this BAP1 relocalization was rapid but transient. BAP1 accumulated at sites of microirradiation within a few minutes, but staining intensity quickly declined and was no longer visible after 30 minutes (Fig. 1A). BAP1 recruitment was also observed in U2OS cells expressing GFP-tagged BAP1 (Fig. 1B). Moreover, placing the GFP tag on either N- or C-terminus of BAP1 did not alter the recruitment of BAP1 to sites of DNA damage (Fig. 1B). Although BAP1 did not form detectable DNA damage foci after cell exposure to ionizing radiation (IR) or genotoxic drugs using different extraction buffers and fixatives (results were summarized in Supplementary Tables S1 and S2), BAP1 displayed enhanced resistance to detergent extraction very early on after treating cells with calicheamicin γ1 (CLM; Fig. 1C), a drug that induces DSBs. CLM binds to the

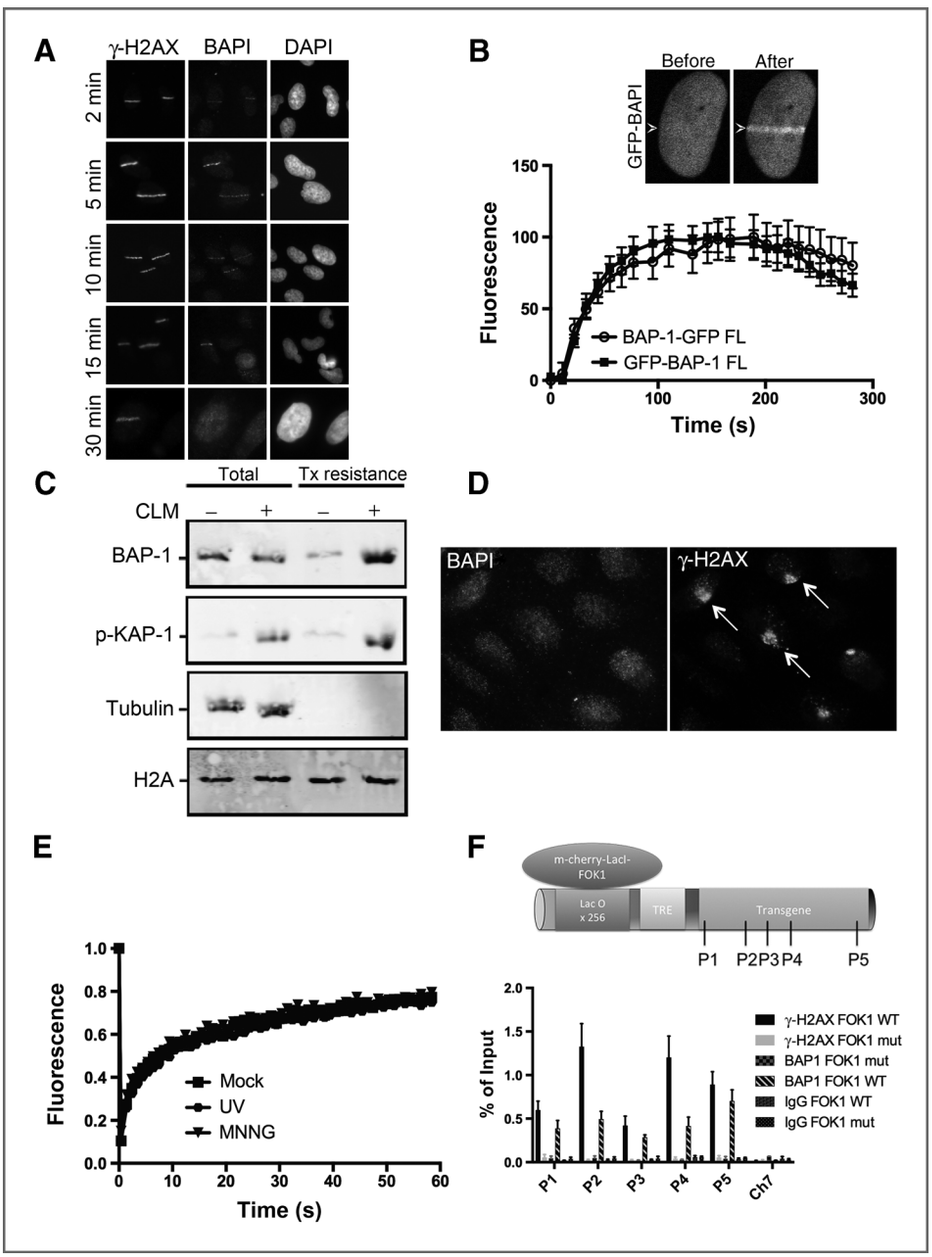

Figure 1. BAP1 is recruited to sites of DNA damage. A, immunodetection of BAP1 and γ-H2AX (damage sites) at the indicated times after laser microirradiation in U2OS cells. B, recruitment of GFP-BAP1 or BAP1-GFP to sites of laser-induced DNA damage after microirradiation in U2OS cells ($n = 15$). Representative pictures before and after DNA damage are shown (top). C, enhanced binding of BAP1 to damaged chromatin in U2OS cells upon CLM treatment (0.1 nmol/L for 60 minutes) analyzed by Western blot analysis with the indicated antibodies. Tx, detergent preextraction with Triton X-100 as described in Materials and Methods. D, immunodetection of BAP1 and γ-H2AX 20 minutes after UV irradiation through a porous membrane. E, FRAP analysis of GFP-BAP1 in U2OS cells either left untreated or treated with UV (60 J/m^2), MNNG (0.1 mmol/L). F, top: schematic of Fok1-induced DSBs reporter system and the positions of the ChIP-qPCR primer sets; bottom: U2OS cells stably expressing the reporter locus were transfected with WT Fok1 (FOK1 WT) or catalytically inactive FOK1 mutant (FOK1 mut) endonuclease constructs. ChIP was performed with IgG, BAP1, and γ-H2AX antibodies. Quantitative PCR using five representative primers was done and the Fok1 DSB-induced enrichment for each primer and antibody is plotted. Error bars represent SEM of at least two independent experiments.

minor groove in the DNA and induces DSBs by two radical centers present in the molecule (42). These data are consistent with the rapid recruitment of BAP1 to damaged chromatin. Because laser microirradiation can induce several types of DNA damage, we also tested the potential of the other major types of damage, UV, and single-strand breaks (SSB), to recruit BAP1. We placed a polycarbonate membrane with 10 μm pores over cells and exposed the cells to UV (1 J/s) for 2 minutes. We

were unable to detect any BAP1 enrichment at sites of UV damage (Fig. 1D and Supplementary Tables S1 and S2). We also performed FRAP experiments to determine if treating cells with UV or an alkylating agent, MNNG, altered the mobility of BAP1, reflecting its retention at sites of DNA damage. We found that neither UV (60 J) nor MNNG (0.1 mmo/L) affected the kinetics of BAP1 (Fig. 1E). We were therefore able to conclude that BAP1 is unlikely to be recruited directly by SSBs or UV damage but may bind DSBs.

BAP1 recruits to Fok1 endonuclease-induced DSBs

To extend our observations, we sought to determine whether BAP1 is recruited to a single DSB. To do so, we took advantage of a previously established DSB reporter system that uses a *Lac*I-*Fok*I nuclease fusion protein to create DSBs within a single genomic locus in U2OS cells (U2OS-DSB reporter; Fig. 1F; ref. 39). ChIP for BAP1 and γ-H2AX at the U2OS-DSB reporter locus produced results consistent with indirect IF staining, showing 2- to 4-fold enrichment of BAP1 and γ-H2AX levels up on induction of DSBs by Fok1 (Fig. 1F and Supplementary Fig. S1B). These results complement the laser microirradiation data and demonstrate that DSBs are among the types of damage that recruit BAP1.

The UCH domain and BAP1 activity are required for BAP1 retention at sites of DNA damage

The WT BAP1 is a nuclear localized DUB with an N-terminal ubiquitin C-terminal hydrolase (UCH) domain, a C-terminal BRCA1 interacting domain (BID), and two predicted nuclear localization signals (NLS; Fig. 2A; ref. 24). We mapped the domains required for its localization to sites of DNA damage. We monitored the recruitment of GFP-tagged BAP1 mutants to sites of DNA damage using time-lapse microscopy (Fig. 2A). An active site point mutant, C91A, which has been shown to have no ubiquitin hydrolase activity, was also tested (24). To overcome the problem that some truncated BAP1 mutants were localized into the cytoplasm, an NLS sequence was added to these BAP1 truncated mutants. We found that ΔBID and 1–393 each had reduced recruitment to sites of DNA damage (Fig. 2B). In contrast, ΔUCH and catalytically inactive BAP1 C91A mutants failed to localize to laser microirradiated tracks. This demonstrates that the catalytic activity and UCH domain of BAP1 are important for recruitment to the DNA damage sites. This observation was confirmed by the ChIP analysis performed in U2OS-DSB reporter cell line transfected with GFP alone, GFP-BAP1 WT or GFP-BAP1-ΔUCH constructs (Supplementary Fig. S2A).

We next assessed the effect of cancer-associated BAP1 mutants (A95D, G178V, C91W, F81V, and S63C) on BAP1 recruitment at DSBs (24, 35, 37). G178V and A95D are point mutations near the BAP1 active site that have been observed in lung cancer and a malignant pleural mesothelioma, and both have also been shown to have reduced ubiquitin hydrolase activity (43). The point mutant F81V was also found to have reduced catalytic activity and is predicted to have a high probability of altering BAP1 deubiquitylase activity (35). A second missense active site point mutant is C91W, which was found to decrease the BAP1 DUB activity using ubiquitin cleavage assay (26). Finally, S63C, which has been shown to have increased catalytic activity compared with WT BAP1, was also examined (37). We laser microirradiated U2OS cells expressing GFP-tagged constructs of these point mutants and examined their recruitment to sites of DNA damage. We found that A95D, G178V, F81V, and C91W each had reduced recruitment to the laser microirradiation induced DNA damage (Fig. 2C) with A95D being the most impaired. It is interesting that one of the mutants known to increase the catalytic activity of BAP1, S63C, retained the same ability to relocalize to the damaged area as the WT protein (Fig. 2C). In a control experiment, we showed that all the GFP-BAP1 constructs expressed to a similar degree (Fig. 2D). These results indicate that BAP1 recruitment at sites of DNA damage involves its catalytic domain.

BAP1 mediates ASXL1 recruitment to sites of DNA damage

The PR-DUB complex consists of BAP1 and additional sex combs–like 1 (ASXL1; ref. 30). ASXL1 was also detected at sites of laser-induced damage (Fig. 3A). Moreover, BAP1 knockdown by short-hairpin RNA (shRNA) treatment impaired ASXL1 recruitment to damaged regions (Fig. 3B). In contrast, ASXL1 knockdown did not impair BAP1 enrichment at sites of laser-induced DNA lesions (Fig. 3C). In a control experiment, we showed that each shRNA depleted the cellular levels of BAP1 and ASXL1 and knocking down of either ASXL1 or BAP1 did not affect the levels of the other protein in cells (Supplementary Fig. S2B and S2C). We also found that IR does not alter the stability of the PR-DUB complex (Supplementary Fig. S2D). Collectively, these findings suggest that the PR-DUB complex is recruited to damage sites.

BAP1 localization to DNA breaks is dependent upon PARP activity

We next sought to determine the mechanism of BAP1 accumulation at sites of DNA damage. Initially, we focused on the potential involvement of H2AX phosphorylation (γ-H2AX) because this histone modification has previously been implicated in the recruitment of several DDR factors to damaged chromatin (reviewed in ref. 44). However, BAP1 accumulation at DNA damage sites was not impaired in H2AX knockout cells (Fig. 3D). We then tested the potential contribution of 2 important enzymes involved in the early steps of the DDR, ATM, and poly(ADP-ribose) polymerase-1 (PARP1). The use of the ATM inhibitor KU-55933 and A-T fibroblasts revealed that ATM activity was dispensable for BAP1 recruitment to damage sites (Supplementary Fig. S3A and S3B). In a control experiment, we show that KU-55933 completely inhibited ATM activity under this experimental condition (Supplementary Fig. S3C). In striking contrast to the above data, chemical inhibition of the PARP family members PARP1 and PARP2 with the potent inhibitor AG14361 or PARP1/2 depletion by RNA interference completely abrogated BAP1 accumulation at sites of laser-induced damage (Fig. 3E and F). In control experiments, we showed that each siRNA efficiently knocked down the levels PARP1/2 in cells (Supplementary Fig. S4A and S4B). In addition, we found that ASXL1 recruitment to

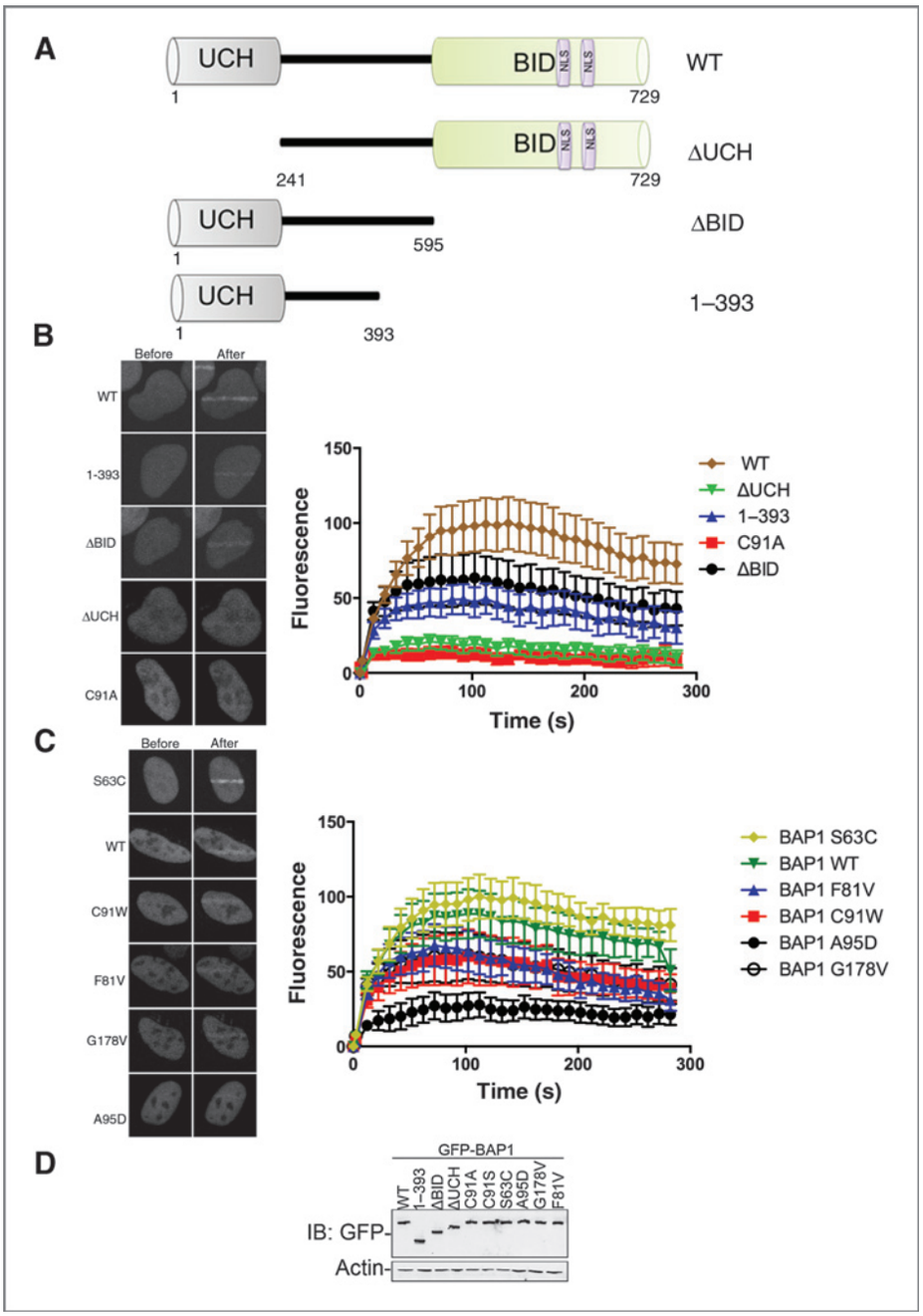

Figure 2. The UCH domain of BAP1 is required for its accumulation at DNA damage sites. A, schematic representation of BAP1 deletion constructs used in B and C. B and C, recruitment of GFP-BAP1 or the individual GFP-tagged BAP1 deletion constructs shown in A to sites of laser-induced DNA damage after microirradiation in U2OS cells ($n = 15$). Representative pictures for each BAP1 construct before and after DNA damage are shown on the left. Error bars represent SEM of at least two independent experiments. D, Western blot analysis of U2OS cells expressing GFP-BAP1 WT and GFP-BAP1 deletion mutants used in B and C.

sites of DNA damage is also impaired upon chemical inhibition of PARP activity in cells (Fig. 3G). However, the dependence of the PR-DUB complex recruitment on PARP activity is indirect because neither BAP1 nor ASXL1 alone or as part of a complex bind PAR polymers using an *in vitro* PAR binding assay (Supplementary Fig. S4C). Taken together, these data suggest that the recruitment of PR-DUB complex to sites of DNA damage requires PARP enzymatic activity but it does not involve direct binding to PAR polymers.

The RNF8-/RNF168-dependent ubiquitylation cascade regulates the recruitment of DNA damage mediators to DNA breaks by catalyzing the addition of ubiquitin moieties to H2A (6, 11, 13, 14, 45). Moreover, the requirement for the UCH domain in retention of BAP1 could reflect ubiquitin binding. To determine the role of RNF8/RNF168 in BAP1 recruitment to sites of DNA damage, we transfected RNF8 knock out MEFs (RNF8 KO) or RNF168 knock out cells (RNF168 KO) with GFP-BAP1 and monitored the recruitment of BAP1 to sites of DNA damage by time-lapse microscopy. We found that RNF8/RNF168 are required for BAP1 recruitment to sites of DNA damage (Fig. 3H). We also tested the requirement for ubiquitylation by pretreating cells with the proteasome inhibitor

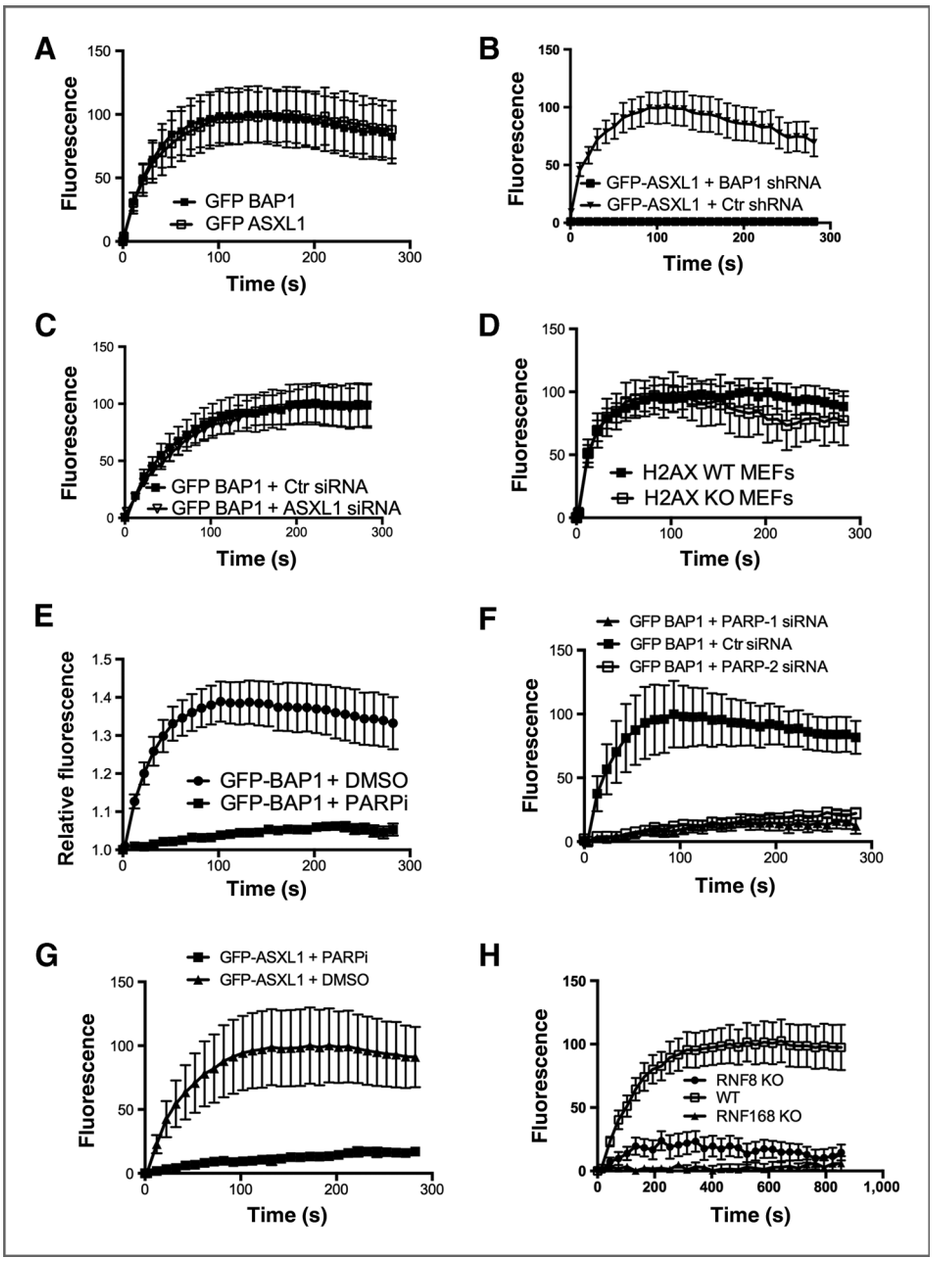

Figure 3. PR-DUB proteins' recruitment to sites of laser-induced DNA damage is PARP-dependent. A, recruitment of GFP-BAP1 and GFP-ASXL1 in U2OS cells after laser microirradiation. B, recruitment of GFP-ASXL1 after laser microirradiation in U2OS cells transfected with the indicated shRNAs. C, recruitment of GFP-BAP1 after laser microirradiation in U2OS cells transfected with the indicated shRNAs. Recruitment of GFP-BAP1 to sites of DNA damage in H2AX WT and H2AX knock out MEFs (H2AX KO; D). E, U2OS cells treated with PARP-1/2–specific inhibitor (PARPi). F, U2OS cells transfected with the PARP 1/2 siRNAs. G, recruitment of GFP-ASXL1 after laser microirradiation in U2OS cells treated with PARPi. H, GFP-BAP1 recruitment in WT, RNF8 KO, and RNF168 KO cells. Error bars represents SEM of two independent experiments ($n = 13$).

MG132. This treatment depletes free ubiquitin and consequently inhibits ubiquitylation at sites of DNA damage (Supplementary Fig. S5A). A recent report showed that PARP mediates RNF168-dependent signaling and repair of DSBs by recruiting SMARCA5, a chromatin remodeling protein that facilitates the recruitment of RNF168 (46) providing one potential mechanism to explain the PARP and RNF168 requirements that we observe.

BAP1 is phosphorylated upon DNA damage in an ATM-dependent manner

A hallmark of many DDR proteins is their posttranslational modification in response to genotoxic stress. Proteomic screens in human cells have identified BAP1 as a target for DDR kinases (47). We found that BAP1 was phosphorylated when cells were exposed to IR (Fig. 4A). Furthermore, this BAP1 phosphorylation was prevented when cells were incubated with wortmannin or the ATM inhibitor KU-55933 but not inhibited when cells were treated with DNA-PKcs inhibitor (DNA-PKi; Fig. 4A). Importantly, detection of BAP1 phosphorylation was abrogated when S592, but not other SQ motifs, was substituted to alanine (Fig. 4B). Consistent with our finding that ATM activity was dispensable for BAP1 accumulation at DNA damage sites, mutation of all S/TQ motifs, including S592, did not interfere with BAP1 recruitment (Fig. 4C). Collectively, these results show that BAP1 is

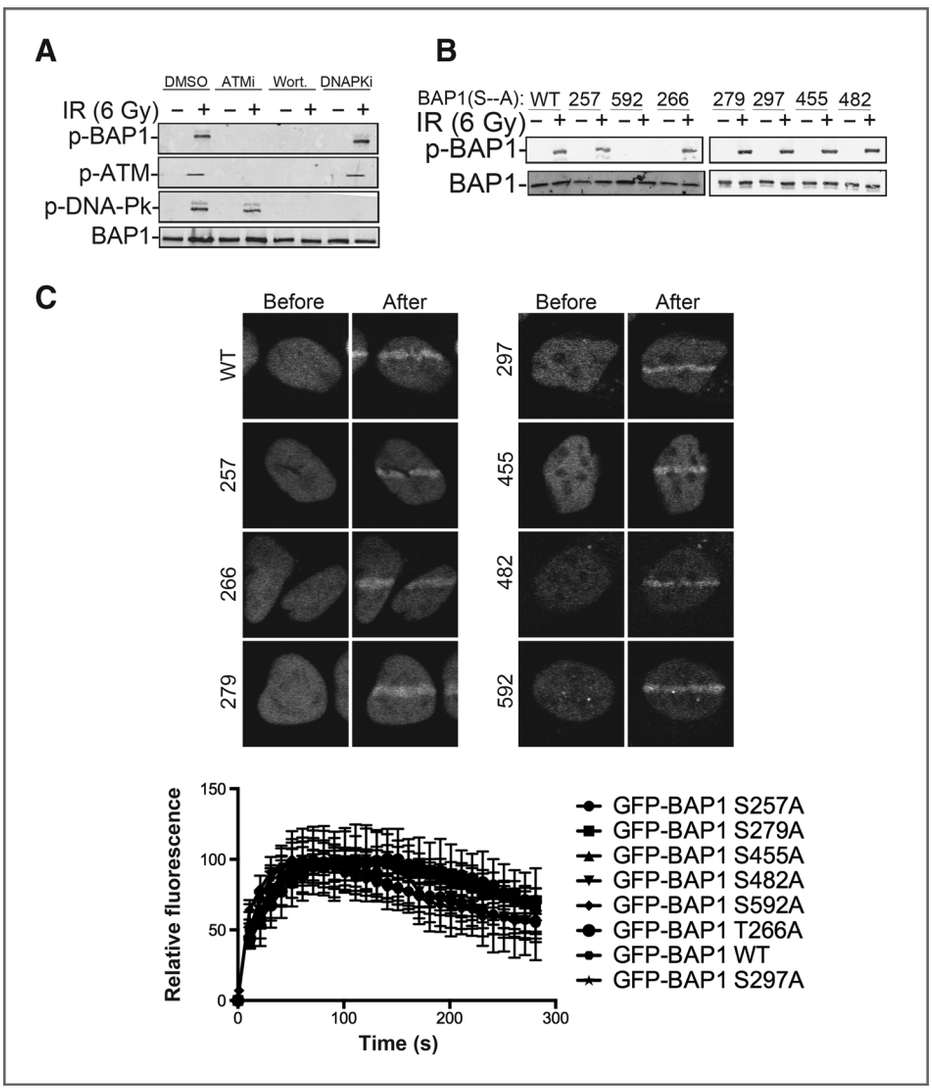

Figure 4. ATM-dependent phosphorylation of BAP1 Ser-592 in response to DNA damage. A, U2OS cells were incubated with the indicated inhibitors (20 μmol/L Wort., Wortmannin; 2 μmol/L DNA-PKi, DNA-PK inhibitor; 10 μmol/L ATMi, ATM inhibitor) for 1 hour and then exposed to IR (6 Gy). Nuclear extracts were isolated and analyzed for the indicated proteins. ATM autophosphorylation on S1981 (p-ATM) and DNA-PKcs autophosphorylation on S2056 (p-DNA-Pk) are used as controls. B, H226 cell lines expressing GFP-BAP1 WT or GFP-BAP1 phosphorylation mutants were exposed to IR (6 Gy). Western blot analysis was conducted with BAP1 and p-BAP1 (pS592) antibodies. C, top, representative pictures of GFP-BAP1 mutants before and after DNA damage are shown; bottom, recruitment of GFP-BAP1 predicted S/TQ point mutants is shown ($n = 14$). Error bars represent SEM of two independent experiments.

phosphorylated after DNA damage on S592 in an ATM-dependent manner and establish that ATM-dependent BAP1 phosphorylation and PAR-dependent BAP1 recruitment to damaged chromatin are distinct events.

BAP1 negatively regulates DSB-induced H2A ubiquitylation

It has been reported that H2A ubiquitylation of lysine residue K119 and lysine residues K13/15 are important in the DSB repair pathway (8, 48, 49). The BAP1 ortholog in *Drosophila melanogaster*, Calypso, deubiquitylates Ub-H2AK119 (30). Therefore, we first determined whether Ub-H2AK119 is a direct substrate for the DUB activity of BAP1 *in vivo*. Overexpression of WT BAP1 but not catalytic dead mutant BAP1 (C91A) reduces Ub-H2AK119 (Fig. 5A, $P = 0.07295$). Furthermore, when BAP1 was depleted by shRNA, Ub-H2AK119 was increased in both the presence and absence of IR (Fig. 5B, $P = 0.022606$). These data indicated that BAP1 is a DUB for Ub-H2AK119. To further confirm these results, we performed a ChIP experiment using a targeted Fok1 endonuclease in U2OS cells. ChIP analysis revealed that induction of a DSB increased Ub-H2AK119 and γ-H2AX occupancy in flanking chromatin (Fig. 5C, $P = 0.00781$). Conversely, whereas BAP1 knockdown did not affect γ-H2AX at sites of damage, depletion of BAP1 resulted in an increase in Ub-H2AK119 accumulation (Fig. 5D, $P = 0.0258$).

BAP1 mediates DSB repair via HR pathway

DSB repair involves 2 major pathways, NHEJ and HR. BAP1 was shown to interact with BRCA1 in overexpression studies. Given that BRCA1 is a key player in DSB repair by HR, we decided to examine the role of BAP1 in HR repair. We used U2OS-DR cells containing a single-copy integrated tandem GFP-based reporter of HR (50) after shRNA-mediated knockdown of BAP1 (Fig. 6A). The generation of a DSB by expression of I-*Sce*I restriction enzyme resulted in 7.4% of the cells becoming GFP-positive cells as a result of HR repair, but BAP1 knockdown by shRNA

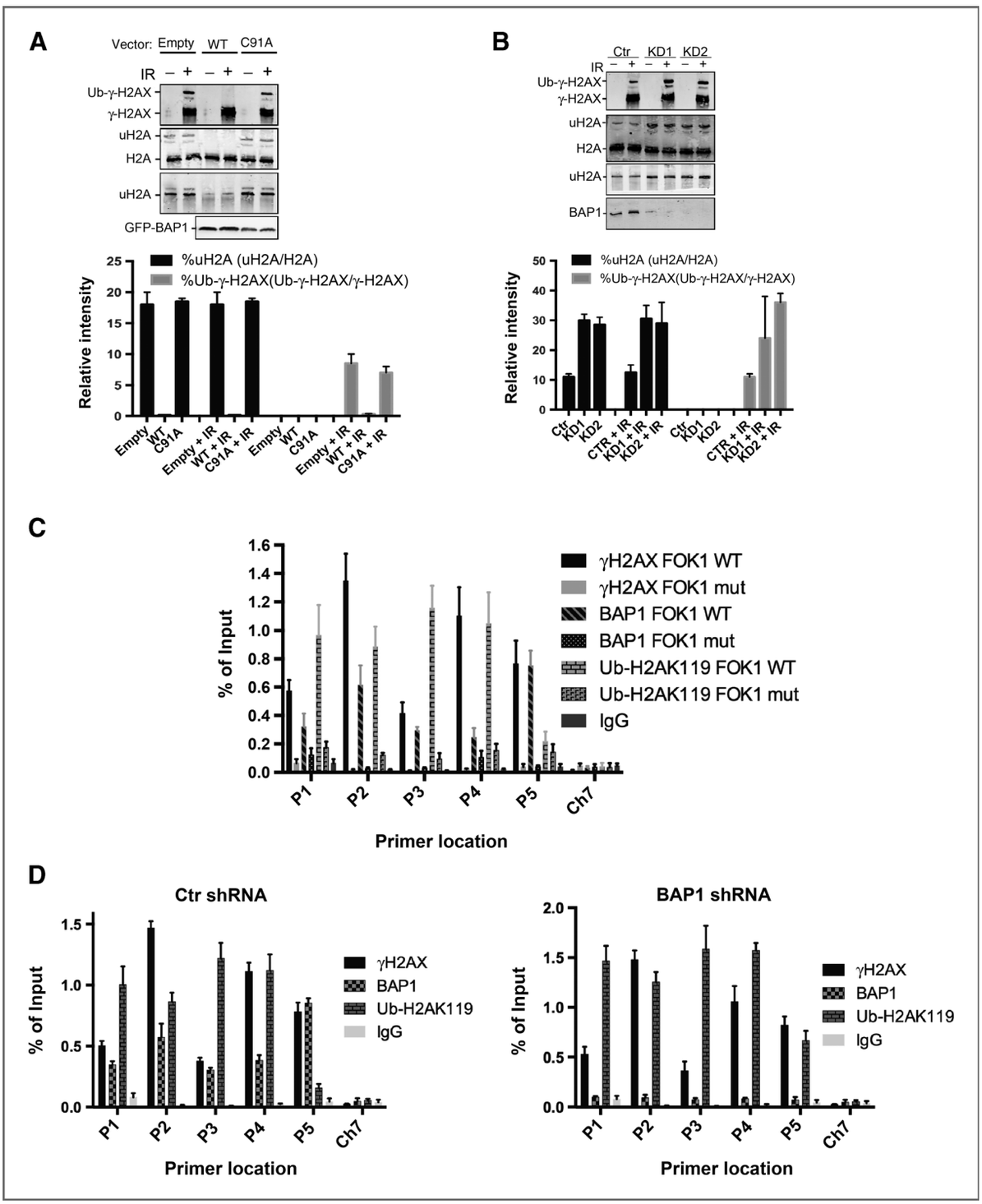

Figure 5. BAP1 regulates H2A de-ubiquitylation at the sites of DNA damage. A, U2OS cells were transfected with GFP alone, GFP-BAP1 WT, or GFP-BAP1 C91A constructs for 24 hours. Cells were then irradiated, and nuclear and acid extracts were prepared and immunoblotted as indicated. B, top, U2OS cells were transfected with either control (Ctr) shRNA or 1 of 2 different BAP1 shRNA constructs (KD1 and KD2) for 48 hours. Cells were then irradiated, and nuclear and acid extracts were prepared and immunoblotted as indicated. Bottom, quantifications of the uH2A and ub-γ-H2AX relative to total H2A and γ-H2AX, respectively, were done with Image J software from at least two different experiments and plotted as indicated. C, ChIP was performed as in Fig. 1F with IgG, Ub-H2AK119, BAP1, and γ-H2AX antibodies. Quantitative PCR using six representative primers was done, and the Fok1 DSB-induced enrichment for each primer and antibody is plotted. D, experiment was done as in C, except cells were cotransfected with either control (Ctr) shRNA or BAP1 shRNA.

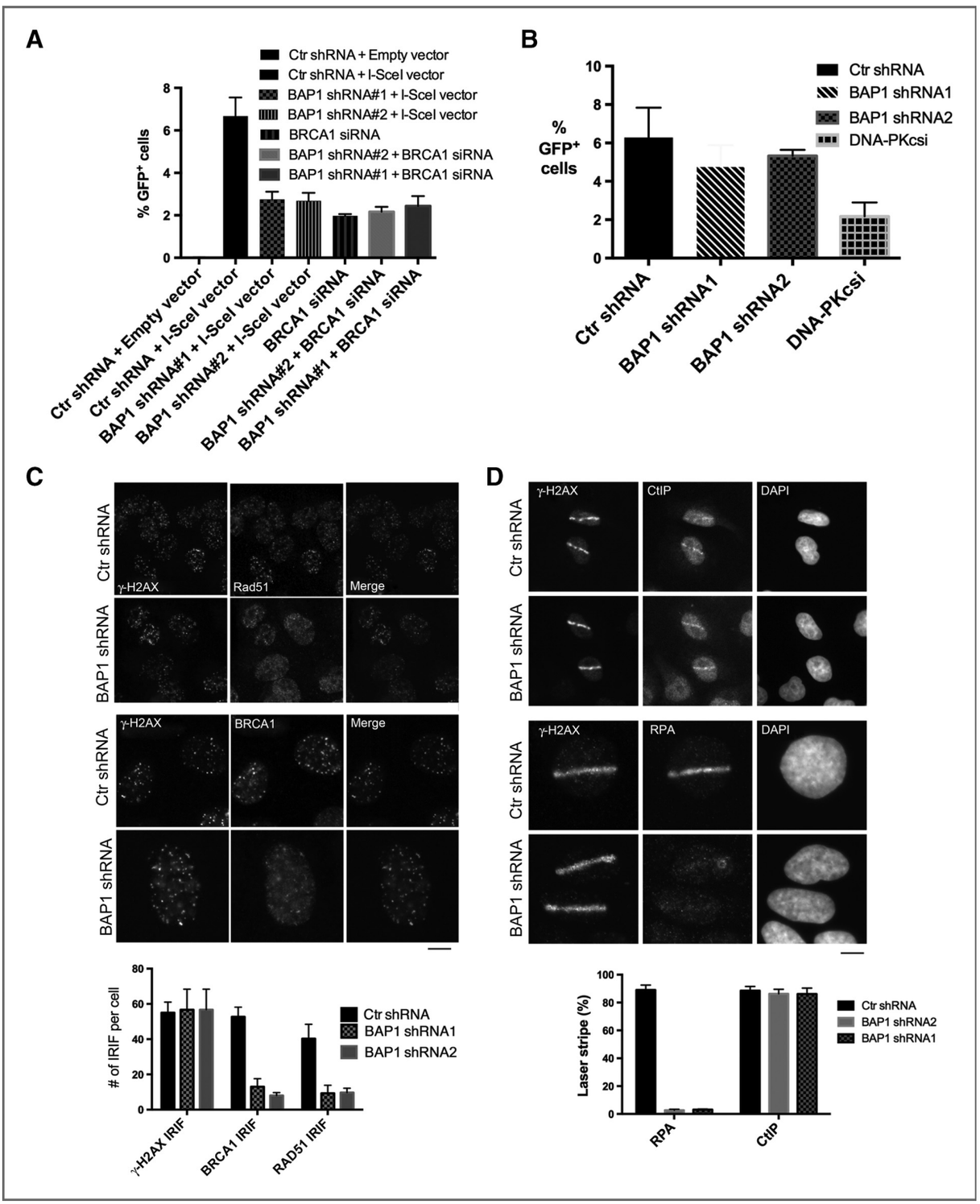

Figure 6. BAP1 promotes HR repair. Efficiency of DSB repair in cells transfected with control (Ctr) shRNA or 2 different BAP1-specific shRNA. A, cells contain an integrated tandem GFP reporter of HR. The percentage of GFP-positive was measured by flow cytometer. Each data point represents the mean ± the SEM of 2 separate experiments. B, cells contain an integrated tandem GFP reporter of NHEJ. The percentage of GFP-positive was measured as in A. C, effect of BAP1 depletion on HR proteins. Cells were treated as in A, irradiated with 2 Gy, and allowed to recover for 4 hours and immunostained with different antibodies as indicated. Representative pictures are shown (top). The average number of foci per cell is shown (bottom). D, the effect of BAP1 depletion on DNA end resection. Cells were treated as in A and were laser microirradiated and immunostained with different antibodies as indicated. Representative pictures are shown (top). Quantification of the relative intensity of the laser stripe is shown (bottom).

decreased this activity by approximately 3-fold (Fig. 6A and Supplementary Fig. S5B, $P = 0.00717$). This decrease in HR activity in cells upon BAP1 knockdown is not fully explained by cell-cycle changes. BAP1 knockdown slightly decreased the S-phase population of the DR-GFP cells (65% G_1, 12% S, and 14% G_2–M phase cells in Ctr cells vs. 69% G_1, 11% S, and 12% G_2–M and 68% G_1, 11% S, and 13% G_2–M in BAP1 KD cells) under the same experimental conditions tested. As BRCA1 is an important factor for HR repair, siRNA-mediated BRCA1 knockdown also shows a low frequency of GFP-positive cells (approximate 2%; Fig. 6A and Supplementary Fig. S6A and S6B, $P = 0.00741$). Knockdown of both BAP1 and BRCA1 simultaneously in cells did not further decrease this HR frequency, suggesting that BAP1 could function for HR repair in a BRCA1-related pathway. In contrast, the effect on DSB repair by NHEJ did not reach significance ($P = 0.1230$), suggesting that BAP1 is less important for NHEJ repair (Fig. 6B and Supplementary Fig. S5B). Cells treated with 2 μmol/L DNA-PKcs inhibitor showed a 60% reduction in the percentage of GFP-positive cells and served as a positive control.

As BAP1 was required for effective HR, we investigated whether BAP1 depletion influenced the accumulation of the HR components RAD51 and BRCA1 at DNA damage sites. IR-induced focus formation of HR proteins such as RAD51 and BRCA1 was reduced in BAP1 knockdown cells (Fig. 6C). The effect of BAP1 depletion on HR components is specific because BAP1 did not inhibit 53BP1 or BMI1 foci formation (Supplementary Fig. S6C).

Resection of DSB ends is one of critical initial reactions for HR repair pathway, and this resection step can be visualized by replication protein A (RPA) and CtIP recruitment to sites of DNA damage. We observed that the relocation of RPA to DNA damage sites was reduced in BAP1 knockdown cells (Fig. 6D). In contrast, BAP1 knockdown did not alter CtIP recruitment to the sites of breaks. We conclude that BAP1 might participate in HR directly, perhaps by facilitating the initial recruitment/accumulation of key HR proteins such as RPA, RAD51, and BRCA1 at DSB sites.

Because functional defects in the BRCA1-mediated DSB repair pathway confer synthetic lethality to PARP inhibition, we performed clonogenic assays using the PARP inhibitor veliparib. We observed increased sensitivity to a PARP inhibitor in U2OS cells when BAP1 levels were reduced by treatment with BAP1 shRNA (Fig. 7A, $P = 0.01472$ at 50 μmol/L). These data are consistent with the differential sensitivity of a renal cell carcinoma cell line (BAP1-deficient cells) to PARP inhibition (51).

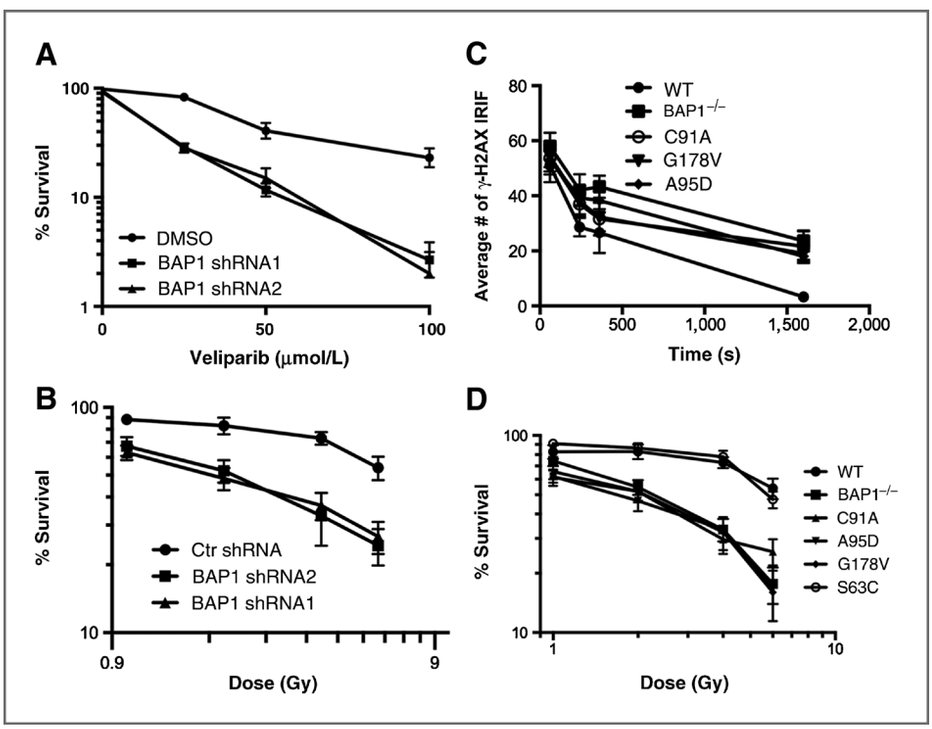

Figure 7. Clonogenic survival of H226 cells transfected with WT BAP1 construct or BAP1 point mutants found in human cancers. A, sensitivity of BAP1 knockdown cells to PARP inhibition. Survival of U2OS cells upon BAP1 knockdown (BAP1 shRNA1&2) compared with DMSO in response to different concentrations of PARP inhibitor (Veliparib). Error bars indicate SEM from two independent experiments. B, clonogenic survival of U2OS cells upon BAP1 knockdown (BAP1 shRNA1&2) compared with control (Ctr shRNA) in response to IR. Error bars indicate SEM from two independent experiments. C, γ-H2AX foci formation in H226 cells or H226 cells reconstituted with different BAP1 mutants at the indicated time points after exposure to 2 Gy IR. γ-H2AX foci were counted and plotted versus time. D, clonogenic survival of H226 cells reconstituted with WT BAP1 or BAP1 point mutants in response to IR. Error bars indicate SD from two independent experiments. The results are normalized to plating efficiency.

Germline BAP1 mutations inhibit DSB repair and increase radiation sensitivity

We used a colony formation cell survival assay to determine if BAP1 plays a role in determining cell fate following DNA damage. We found that BAP1 knockdown resulted in hypersensitivity to IR (Fig. 7B, $P = 0.00313$ at 2 Gy). We next investigated whether mutations in BAP1 reported to occur in cancer alter the ability of these cells to resolve DNA damage. A previously described malignant pleural mesothelioma (MPM) cell line (H226) lacking BAP1 expression (37) or H226 cells reconstituted with WT or mutant BAP1 constructs were exposed to 2 Gy of IR. Cells were allowed to recover at 37°C for 2, 4, 8, or 24 hours and then stained for γ-H2AX to evaluate DSB repair efficiency by counting the number of γ-H2AX foci per cell over time. We found that BAP1-deficient cells have slower repair kinetics compared with cells that have normal expression of BAP1 (Fig. 7C, $P = 0.00784$ at 24 hours). Consistent with the importance of the catalytic activity of BAP1, we found that BAP1-null cells reconstituted with BAP1 mutants C91A, A95D, or G178V are unable to efficiently repair DSBs (Fig. 7C, $P = 0.01217$ at 24 hours) or correct the observed sensitivity defect of BAP1-null cells (Fig. 7D, $P = 0.00968$ at 6 Gy). We also observed that reconstitution of BAP1-deficient cells with the catalytically active BAP1 S63C mutant gives a survival advantage (Fig. 7D, $P = 0.01831$ at 2 Gy). Collectively, these findings may help explain recent observations linking BAP1 to cancer-related syndromes.

Discussion

BAP1 is the catalytic subunit of the PR-DUB transcriptional repressor complex (30). Recent studies have shown that in 42% of MPM, an aggressive neoplasm of the serosal lining of the chest, there was a loss or mutation (or both) of BAP1(37). In addition, germline mutations in BAP1 have been shown to predispose individuals to melanocytic tumors. In this study, we describe the participation of BAP1 in the regulation of DSB signaling and efficient DSB repair. This provides a mechanism for BAP1 to directly contribute to genome stability. Specifically, we have shown that BAP1 is an important contributor to multiple aspects of the DDR. BAP1 is phsosphorylated by ATM in response to DNA damage and recruits to DNA damage sites together with ASXL1 in a PARP and RNF8/RNF168-dependent manner. BAP1 deubiquitylates Ub-H2AK119 at sites of DNA damage and loss of BAP1 results in impaired HR repair and increased sensitivity to radiation and PARP inhibitors.

BAP1 was identified in a yeast 2-hybrid screen for proteins that interacted with BRCA1 (23). Because BRCA1 is a key HR protein, it was predicted that autoubiquitylated BRCA1 might be a substrate for BAP1 DUB activity and BAP1 may play a role in the DDR (23). Although BAP1 was identified in a proteomic screen as an ATM/ATR substrate that was phosphorylated in response to DNA damage (47), there is presently no evidence that BAP1 functions in the deubiquitylation of the BRCA1/BARD1 complex, leaving its function in DNA repair unclear. We found that BAP1 is required for efficient HR repair and regulates the accumulation of BRCA1, RAD51, and RPA at the sites of DNA damage. BAP1 is recruited to sites of DNA damage in a manner that is dependent on PARP1/2 activity as well as ubiquitylation and the RNF8/RNF168 E3 ubiquitin ligases. Although we observe a PARP-dependent recruitment of BAP1, our data suggest that BAP1 does not directly interact with PAR polymers. Interestingly, a recent report showed that PARP regulates SMARCA5, which directly regulates the ubiquitin response by promoting RNF168 accumulation at DSBs and subsequent ubiquitin signaling (46). The simplest explanation for our results, then, is that BAP1 recruited through ubiquitin deposited by RNF168 and is sensitive to PARP inhibition because PARP inhibition impairs RNF168 recruitment.

Several mechanisms might explain how BAP1 regulates HR proteins. The BRCA1 E3 ubiquitin ligase activity is important in BRCA1's function in HR repair because BRCA1 transgenes with E3 ligase mutations are unable to restore HR in BRCA1-defective cells using I-*Sce*I based HR reporters (52, 53). Thus, it is possible that BAP1 mediates deubiquitylation of BRCA1 substrates, including BRCA1 autoubiquitylation. Alternatively, BAP1 might deubiquitylate a substrate, such as histone H2A, that is required for BRCA1-mediated function in HR repair. Our data showed that BAP1 negatively regulates H2AK119 ubiquitylation. Interestingly, histone H2A ubiquitylation has been shown to facilitate a DSB-mediated transcription block, a step that mediates DSB repair and heterochromatin-mediated silencing (39, 54). A recent study showed that BAP1 is phosphorylated at S592 in S-phase following replication stress (55). Interestingly, HR is the main pathway that repairs DSBs during S-phase. Given the new function of BAP1 in HR that we describe, it is possible that phosphorylation of BAP1 at S592 is a regulatory mechanism to control the function of BAP1 in HR repair. Further studies will be required to test this possibility.

In experiments performed with renal cell carcinoma cell lines, BAP1 loss was associated with increased sensitivity to PARP inhibitor olaparib (51). In mesothelioma cell lines, no difference was observed between BAP1-mutant and WT cells in sensitivity to PARP inhibitors (37). We observed differential sensitivity of BAP1 knockdown cells to PARP inhibitor, Veliparib, which is similar to the sensitivity of BRCA1-deficient cells to PARP inhibition. The synthetic lethality of BAP1-deficient cells to PARP inhibition is likely because of its role in HR repair. Consistent with this, BAP1 depletion impairs the assembly of RPA, RAD51, and BRCA1 at DNA damage sites. Depleting both BAP1 and BRCA1 did not further sensitize cells. These results are consistent with a function of BAP1 in BRCA1-mediated processes.

We found that mutations observed in cancers that decreased the catalytic activity of BAP1 impaired the recruitment of BAP1 to sites of laser microirradiation. Consistent with this finding, a point mutant that increased catalytic activity (S63C) recruited to similar levels as WT BAP1. This suggests that catalytic activity is necessary for efficient recruitment to the sites of DSB. It also suggests a broader function for BAP1 in tumorigenesis. For example, mutants

that cause either a decrease in or loss of catalytic activity are associated with oncogenesis in MPM. Consistent with this notion, we found that an MPM cell line lacking BAP1 expression (H226) or reconstituted with BAP1 germline mutants was unable to efficiently repair DSBs. This repair defect is consistent with the observed radiation sensitivity of BAP1-null cells and BAP1-null cells reconstituted with BAP1 germline mutations. Given the spectrum of neoplasms associated with these BAP1 germline mutations, these data suggest that BAP1 has an important tumor-suppressive function in multiple tissues. Our results indicate that alterations in the equilibrium between ubiquitylation and deubiquitylation disrupt DSB repair and thus may lead to genomic instability, a hallmark of cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): I.H. Ismail, R. Davidson, J.-P. Gagné
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): I.H. Ismail, R. Davidson, G.G. Poirier, M.J. Hendzel
Writing, review, and/or revision of the manuscript: I.H. Ismail, R. Davidson, J.-P. Gagné, M.J. Hendzel
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R. Davidson
Study supervision: I.H. Ismail, G.G. Poirier, M.J. Hendzel
Other (generated all the BAP1 and ASXL1 point mutation and truncated constructed constructs used in the study): Z.Z. Xu

Acknowledgments

The authors thank Drs. S. Janicki, J. Stark, R. Greenberg, and K.D. Wilkinson for providing valuable reagents. They also thank the Cellular Imaging Facility and Flow Cytometry Units at the Cross Cancer Institute for the use of microscopes and flow cytometers.

Grant Support

This work was supported by an operating grant from the Canadian Institute of Health Research (CIHR).

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Received November 4, 2013; revised May 27, 2014; accepted May 28, 2014; published OnlineFirst June 3, 2014.

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