The Deubiquitylating Enzyme USP44 Counteracts the DNA Double-strand Break Response Mediated by the RNF8 and RNF168 Ubiquitin Ligases*†

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Background: The RNF8/RNF168 E3 ligase cascade promotes ubiquitin-dependent protein assembly at DNA double-strand breaks (DSBs).

Results: An overexpression screen identified new deubiquitylating enzymes (DUBs) opposing the RNF8/RNF168 pathway in human cells.

Conclusion: USP44 counteracts RNF168-dependent ubiquitylation of proteins at DSB sites, including histone H2A.

Significance: Identification of antagonizers of the RNF8/RNF168 pathway is crucial for understanding how cells regulate DSB repair.

Protein recruitment to DNA double-strand breaks (DSBs) relies on ubiquitylation of the surrounding chromatin by the RING finger ubiquitin ligases RNF8 and RNF168. Flux through this pathway is opposed by several deubiquitylating enzymes (DUBs), including OTUB1 and USP3. By analyzing the effect of individually overexpressing the majority of human DUBs on RNF8/RNF168-mediated 53BP1 retention at DSB sites, we found that USP44 and USP29 powerfully inhibited this response at the level of RNF168 accrual. Both USP44 and USP29 promoted efficient deubiquitylation of histone H2A, but unlike USP44, USP29 displayed nonspecific reactivity toward ubiquitylated substrates. Moreover, USP44 but not other H2A DUBs was recruited to RNF168-generated ubiquitylation products at DSB sites. Individual depletion of these DUBs only mildly enhanced accumulation of ubiquitin conjugates and 53BP1 at DSBs, suggesting considerable functional redundancy among cellular DUBs that restrict ubiquitin-dependent protein assembly at DSBs. Our findings implicate USP44 in negative regulation of the RNF8/RNF168 pathway and illustrate the usefulness of DUB overexpression screens for identification of antagonizers of ubiquitin-dependent cellular responses.

The cellular response to DNA double-strand breaks (DSBs) is characterized by rapid accumulation of a large number of repair and signaling factors in the vicinity of the lesions (1–3). Locally, these structures, commonly referred to as ionizing radiation-induced foci (IRIF), protect and sequester the broken DNA ends and modulate a number of cellular processes including transcription, chromatin transactions, and DNA repair. In parallel, IRIF also provide an important checkpoint signaling platform from which DNA damage-activated kinases such as ATM and ATR (ATM and Rad3-related) can globally impact on cell cycle transitions, cell fate decisions, and transcriptional processes (1). Recruitment of numerous factors to the chromatin regions surrounding DSBs requires a ubiquitylation cascade that has been extensively characterized in recent years. Initially, ATM and related kinases phosphorylate H2AX (γ-H2AX), leading to recruitment of the central DDR scaffold protein MDC1, the ATM-mediated phosphorylation of which then provides a docking platform for the RING finger ubiquitin ligase RNF8 (2). Although the full spectrum of RNF8 ubiquitylation targets is likely to be broader than currently appreciated, one central substrate for DNA damage-induced, RNF8-mediated polyubiquitylation is H2A-type histones (4, 5). Although RNF8 seems to play an important role in initiating DSB-induced ubiquitylation of H2A-type histones, it is a second ubiquitin ligase, RNF168, that together with Ubc13 catalyzes bulk DSB-associated histone ubiquitylation. RNF168 is recruited to sites of DNA damage by recognition of RNF8 ubiquitylation products via its three ubiquitin binding domains and specifically ubiquitylates Lys-13 and Lys-15 on H2A-type histones (6–9).

A major consequence of local RNF8/RNF168-mediated ubiquitylation is the retention of genome caretaker factors such as BRCA1 and 53BP1 at sites of DNA damage. Genetic evidence suggests that these proteins engage in a tug-of-war to promote DSB repair by homologous recombination or nonhomologous end joining, respectively (10–12). BRCA1 is attracted to polyubiquitylated histones via its binding partner RAP80, which contains tandem ubiquitin interacting motifs that are optimally spaced for recognition of Lys-63-linked ubiquitin chains (13–17). 53BP1 retention is also likely to require histone ubiquity-
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lation given that overexpression of the H2A deubiquitylating enzyme (DUB) USP3 prevents 53BP1 accrual at DSBs (7). However, unlike RAP80, 53BP1 does not contain obvious ubiquitin-binding motifs, and it is not known to interact with factors that can target it to ubiquitylated core histones. Recently, the accumulation of 53BP1 at sites of DNA damage has been suggested to require the local unmasking of methylated histones, the prime interaction partner of the 53BP1 tandem Tudor domain, catalyzed by RNF8-mediated displacement of L3MBTL1 and/or degradation of JDJM2 family proteins (18, 19).

Although much has been gleaned about the mechanisms that initiate and propagate DSB-induced chromatin ubiquitylation, considerably less is known about the cellular activities that limit, restrain, and reverse this process. Available evidence suggests, however, that such regulatory mechanisms are both complex and multilayered. One way in which cells avoid the detrimental spreading of the DSB-induced ubiquitin signal along chromosome arms is through tight regulation of RNF168 levels by the ubiquitin ligases TRIP12 and UBR5 (20). In addition, RNF168 activity is kept in check by the DUB OTUB1, which binds to Ubc13 in a noncatalytic manner, restricting its interaction with cognate E3 binding partners including RNF168 (21). In addition to mechanisms that restrain DDR-associated histone ubiquitylation by direct regulation of RNF168 activity, several DUBs have been suggested to be catalytically active against RNF8/RNF168-generated ubiquitylation products. These include BRCC36, a BRCA1-associated DUB that has been proposed to edit ubiquitin chains on H2A to facilitate optimal recognition by RAP80; USP16, which was suggested to reverse RNF8/RNF168-mediated transcriptional silencing through H2A deubiquitylation; and USP3, which also deubiquitylates H2A and H2B and suppresses formation of 53BP1 foci when overexpressed (7, 22–24). Finally, POH1, a 19 S proteasome-associated DUB has recently been suggested to cleave Lys-63-linked ubiquitin chains at sites of DNA damage (25).

The relative importance of and interplay between these inhibitory mechanisms for the correct dosing of DSB-associated histone ubiquitylation are poorly understood. Here, we employed an overexpression approach involving the majority of human DUBs predicted to be catalytically active in a search for additional cellular DUBs that can counteract the RNF8/RNF168-dependent histone ubiquitylation pathway. We uncovered USP44 as a powerful new antagonist of RNF8/RNF168-mediated H2A ubiquitylation, which is recruited to ubiquitin-modified DSB-flanking chromatin and may provide an additional regulatory weapon in the cellular arsenal that ensures tight control of DDR-associated histone ubiquitylation.

EXPERIMENTAL PROCEDURES

Plasmids and siRNA—Full-length cDNAs for human USP44, USP29, and USP3 were amplified by PCR and inserted into pEGFP-C1 (Clontech) or pcDNA4/TO (Invitrogen) containing N-terminal Strep-HA or FLAG tags to generate doxycycline-inducible mammalian expression constructs for GFP- and Strep-HA-tagged USP44, Strep-HA-tagged USP29, and FLAG-tagged USP3, respectively. Other human DUBs were cloned into similar expression vectors by PCR-based cloning (supplemental Fig. S1). The USP44 catalytically inactive (CI) (C282A), USP29 CI (C294S), USP3 CI (C168S), and OTUB1 CI (C91S) point mutations were introduced using the QuikChange site-directed mutagenesis kit (Stratagene). All constructs were verified by sequencing. Constructs expressing Myc-ubiquitin and FLAG-H2A were described previously (5). Plasmid transfections were performed using FuGENE 6 (Promega) according to the manufacturer’s instructions. siRNA transfections were done with Lipofectamine RNAiMax (Invitrogen). siRNA target sequences used in this study were: Control (5′-GGGAGUCCUAAGCAGUUCAUA-3′), USP44(#1) (5′-GCAUGUGACACAAAUCAA-3′), USP44(#2) (5′-GACUGACCUCUAAAGAU-3′), USP3 (5′-GGGAGUAAAGGAUCAUAUAU-3′ and 5′-GCGAGUACAGUACAGUACUA-3′), OTUB1 (5′-CCGACUCACCUUGUGGUCUU-3′ and 5′-UGACGGAACUGUUCUAUAU-3′), RNF8 (5′-UGCGGAUAGAAUAGAUGAA-3′), and RNF168 (5′-GGCGAAGAGGCAUGAGGAGA-3′).

Cell Culture—Human U2OS cells were cultured in DMEM containing 10% fetal bovine serum. To generate cell lines constitutively expressing GFP-tagged WT and mutant USP44, U2OS cells were co-transfected with USP44 expression constructs and pBabe.puro, and positive clones were selected with puromycin (26, 27). To generate cell lines inducibly expressing tagged WT and mutant DUB alleles, U2OS cells were co-transfected with DUB expression plasmids and pcDNA6/TR, and positive clones were selected with Zeocin and Blasticidin S, as described (26, 27). To induce DNA double-strand breaks, cells were exposed to X-rays using a Y.SMART tube (YXLON A/S, Taastrup, Denmark) at 6 mA and 160 kV through a 3-mm aluminum filter. Unless stated otherwise, cells were exposed to a dose of 4 Gy and harvested 1 h later.

Immunochemical Methods and Antibodies—Immunoblotting, immunoprecipitation, and Strep-Tactin pulldowns were done as described (28). In vivo H2A ubiquitylation assays were performed as described previously (5). For acid extraction of histones, cells were lysed in PBS containing 0.5% Triton X-100, and nuclei were recovered by centrifugation. Histones were extracted by resuspension of nuclei in 0.2 M HCl for 2 h at 4 °C. Antibodies used in this study included: mouse monoclonal antibodies to Myc (sc-40), GFP (sc-9996), MDM2 (sc-965), and HA (sc-7392) (all from Santa Cruz Biotechnology), FK2 (BML-PW8810, Enzo), H2B-Ub (05-1312) and γ-H2AX (05-636) (both from Millipore), FLAG (F1804, Sigma), and USP3 (H00009960-M01, Abnova); rat monoclonal antibodies to HA (11867423991 and 12013819001, Roche Applied Science); rabbit polyclonal antibodies to 53BP1 (sc-22760), p53 (sc-6243), and proliferating cell nuclear antigen (sc-7907) (Santa Cruz Biotechnology); and rabbit polyclonal antibodies to MCM6 (sc-9843) and GAPDH (sc-20357) (both from Santa Cruz Biotechnology).

Immunofluorescence Staining, Microscopy, and Laser Microirradiation—Cells were fixed in 4% formaldehyde, permeabilized with PBS containing 0.2% Triton X-100 for 5 min, and incubated with primary antibodies diluted in DMEM for 1 h at room temperature. Following staining with secondary antibodies (Alexa Fluor 488 and 568; Invitrogen) for 30 min, coverslips were mounted in VECTASHIELD mounting fluid.
medium (Vector Laboratories) containing the nuclear stain DAPI. Confocal images were acquired on an LSM-780 (Carl Zeiss) mounted on a Zeiss-AxioObserver Z1 equipped with a Plan-Neofluar 40×/1.3 oil immersion objective. Dual and triple color confocal images were acquired with standard settings for excitation of DAPI, Alexa Fluor 488, Alexa Fluor 568, and Alexa Fluor 647 dyes (Molecular Probes, Invitrogen), respectively. Image acquisition and analysis was carried out with LSM-ZEN software. Laser microirradiation of cells was performed essentially as described (28), except that the UV-A laser line used was 355 nm instead of 337 nm. For automated analysis of fluorescence intensities of nuclear foci, exponentially growing U2OS cells were treated with siRNAs (25 nm final concentration) for 3 days. Cells were then irradiated (0.25 Gy), and 30 min later, they were fixed and immunostained with FK2 and 53BP1 antibodies. Nuclear DNA was counterstained with DAPI (0.25 μg/ml). A series of random fields was recorded automatically using the Olympus ScanR imaging work station (Hamamatsu ORCA-R2 camera, UPLSAPO 40×/0.9 objective). The number and intensity of IR-induced nuclear foci were quantified using the ScanR image analysis software.

RESULTS

The Activities of Human USP44, USP29, and USP26 Antagonize Recruitment of 53BP1 to DSB Sites—To identify human DUBs that oppose ubiquitin-dependent protein recruitment to DSB-modified chromatin, we generated an inventory of expression vectors for the majority of human DUBs predicted to be catalytically active (29) (supplemental Fig. S1). We then assessed the ability of individually overexpressed DUBs to counteract IR-induced formation of 53BP1 foci, a process critically dependent on RNF8/RNF168-generated ubiquitylations (2). Among ~60 human DUBs analyzed by this approach, only five were able to consistently and potently abrogate 53BP1 focus formation (Fig. 1A and D; supplemental Fig. S1), indicating that most DUBs do not promiscuously reverse RNF8/RNF168-catalyzed ubiquitylations when expressed at high levels. Of these, USP3 and OTUB1 have previously been described as antagonizers of 53BP1 focus formation (7, 21), confirming the validity of our approach, whereas three additional DUBs capable of preventing 53BP1 recruitment to IRIF, USP44, USP29, and USP26, have not been linked to DDR. Consistent with such an involvement, these proteins all localized exclusively to the nucleus when overexpressed (Fig. 1A; supplemental Fig. S1). USP29 and USP26 are highly homologous proteins; indeed, USP26 is likely a testis-specific homologue of USP29 (29, 30), and we did not pursue this enzyme further in our study. In our screen, a number of DUBs that have been described as H2A DUBs in various cellular contexts (31, 32) did not abrogate 53BP1 focus formation when overexpressed (Fig. 1B). This indicates that the mere ability of a DUB to target mono ubiquitylated H2A is not sufficient to prevent 53BP1 recruitment to DSB sites and/or that the activity of these DUBs may be highly dependent on cofactors or the cellular context.

USP3 is known to directly deubiquitylate H2A, whereas OTUB1 counteracts 53BP1 focus formation in a noncanonical manner involving disruption of the interaction between the E2 enzyme Ubc13 and cognate E3 ligases such as RNF168 (21, 24).

Thus, the ability of OTUB1 to impair 53BP1 recruitment into IRIF did not require its catalytic activity as expected, in contrast to USP44, USP29, and USP3, the CI forms of which all failed to suppress formation of 53BP1 foci (Fig. 1, C and D). We conclude from these experiments that USP44, USP29, and USP26 are novel DUBs whose activities antagonize ubiquitin-dependent recruitment of 53BP1 to DSB-modified chromatin.

USP44 and USP29 Suppress DBS Recruitment of Factors Downstream of RNF8—We next attempted to identify the intervention points of USP44 and USP29 in the RNF8/RNF168-mediated ubiquitylation pathway that promotes 53BP1 recruitment to sites of DNA damage. Using a cell line stably expressing GFP-tagged RNF8, we noted that overexpression of USP44 or USP29 did not interfere with RNF8 accumulation at DSBs (Fig. 2, A and B). In contrast, the ability of cells to concentrate the downstream ubiquitin ligase RNF168 in IR-induced foci was severely compromised in cells overexpressing active USP44 or USP29 (Fig. 2, C and D). This indicates that USP44 and USP29 counteract the ubiquitin-dependent step leading to RNF168 recruitment to DSB sites downstream of RNF8, reminiscent of the effect of knockdown of key regulatory factors including PIAS4 and HERC2, which impair DSB-induced histone ubiquitylation (33–35). Consistently, overexpression of WT but not inactive forms of USP44 and USP29 impaired RNF168-dependent accumulation of ubiquitin conjugates in IRIF (detected by the FK2 monoclonal antibody) (Fig. 2, E and F), as well as focus formation of RAP80 (Fig. 2, G and H), which recruits the BRCA1-A complex to DSB sites through direct recognition of RNF168-generated ubiquitylation products. These findings suggest that USP44 and USP29 reverse DSB-associated ubiquitylations recognized by RNF168, interfering with recruitment of downstream DNA repair factors.

USP44 Promotes Deubiquitylation of Histone H2A—The above observations suggested that elevated activity of USP44 and USP29 might directly counteract H2A ubiquitylation similar to USP3, and we tested this hypothesis by biochemical approaches. To this end, we generated U2OS-based stable cell lines capable of overexpressing USP44, USP29, or USP3 in an inducible manner (Fig. 3A) and performed acid extraction of histones to examine their ubiquitylation status upon ectopic expression of these DUBs. Induction of each of these DUBs caused a prominent loss of monoubiquitylated H2A (Fig. 3A), in agreement with previous literature on USP3 and supporting the notion that USP44 and USP29 similarly suppress 53BP1 focus formation by promoting H2A deubiquitylation. Consistent with recent findings on USP3 and USP44 (24, 36), we also observed a marked decrease in H2B monoubiquitylation upon overexpression of these proteins and USP29 (Fig. 3A), suggesting that each of these DUBs potently reverses ubiquitylation of core histones. To probe for possible pleiotropic effects that may arise from DUB overexpression, we analyzed the propensity of USP44 and USP29 to promote deubiquitylation of other DDR components. The expression levels of the tumor suppressor p53 are tightly regulated by DUBs targeting p53 itself as well as its E3 ligase MDM2 (37). We observed a strong increase in the levels of both p53 and MDM2 in cells overexpressing USP29 (Fig. 3B). In contrast, high levels of USP44 did not impact on the abundance of p53 and MDM2 (Fig. 3B). Likewise, overexpres-
Overexpression of USP44, USP29, and USP26 suppresses IR-induced formation of 53BP1 foci. A, a screen for human DUBs whose overexpression impairs recruitment of 53BP1 to DSB sites. Human U2OS cells were transfected with expression plasmids encoding epitope-tagged human DUBs for 24 h, exposed to IR (4 Gy), and fixed 1 h later. Cells were then co-immunostained with antibodies against the DUB epitope tag (DUB) and 53BP1. Positive hits from the screen are shown. Arrows indicate transfected cells that do not form 53BP1 foci. Scale bar, 10 μm.

B, a siRNA screen, showing human DUBs reported to deubiquitylate H2A that did not suppress 53BP1 foci formation when overexpressed. Images of other human DUBs tested are shown in supplemental Fig. S1. Scale bar, 10 μm.

C, a siRNA screen, except that cells were transfected with CI point mutants of the indicated DUBs. Arrows indicate transfected cells that do not form 53BP1 foci. Scale bar, 10 μm.

D, quantification of the experiments shown in A and C. At least 100 transfected cells per sample were counted. Error bars represent the S.D. of three independent experiments.

We next analyzed how knockdown of USP44 affects H2A ubiquitylation status in cells. As in previous studies (36, 38, 39), we were unable to detect endogenous USP44 with a range of custom-made as well as commercial antibodies, likely due to very low expression levels of this protein. We instead validated the knockdown efficiency of USP44 siRNAs using cells inducibly expressing ectopic USP44 (Fig. 3C). Knockdown of USP44 expression by these siRNAs did not detectably affect levels of

4 A. Mosbech, S. Bekker-Jensen, and N. Mailand, unpublished observations.
monoubiquitylated H2A in interphase cells, similar to what we observed when we removed any of the known H2A DUBs, including USP3 (Fig. 3, D and E; data not shown), suggesting that no single DUB is responsible for limiting bulk cellular levels of monoubiquitylated H2A. We also tested whether USP44 knockdown would impact on the mitosis-associated loss of H2A and H2B ubiquitylation. However, neither USP44 nor USP16, which has previously been implicated in this process (40), significantly interfered with deubiquitylation of core histones in mitosis when knocked down individually or in combination (data not shown). Because USP44 overexpression abrogated H2A ubiquitylation and recruitment of DSB repair

**FIGURE 2.** USP44 and USP29 activity antagonizes recruitment of RNF168 and downstream factors to DSBs. A, U2OS cells stably expressing GFP-RNF8 were transfected with WT or CI versions of Strep-HA-tagged USP44 and exposed to IR (4 Gy). Cells were fixed 1 h later and immunostained with HA antibody. B, as in A, except that cells were transfected with Strep-HA-USP29 plasmids. C, U2OS cells transfected with Strep-HA-USP44 constructs and exposed to IR (4 Gy) were fixed 1 h later and co-immunostained with RNF168 and HA antibodies. D, U2OS cells transfected with Strep-HA-USP29 constructs and exposed to IR (4 Gy) were fixed 1 h later and co-immunostained with RNF168 and HA antibodies. E, as in C, except that cells were co-immunostained with FK2 and HA antibodies. F, as in D, except that cells were co-immunostained with FK2 and HA antibodies. G, as in C, except that cells were co-immunostained with RAP80 and HA antibodies. H, as in D, except that cells were co-immunostained with RAP80 and HA antibodies. All Scale bars, 10 μm.
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Factors to damaged chromatin, we reasoned that USP44 might primarily target H2A in a more context-dependent manner involving the reversal of its DSB-associated polyubiquitylation by RNF168. Hence we asked whether USP44 could target ubiquitin chain-modified H2A, a hallmark of RNF168 activity. To this end, we ectopically co-expressed H2A and ubiquitin to facilitate visualization of oligo- and polyubiquitylated forms of H2A (5) and tested how overproduction of active or inactive forms of USP44 affected such modifications. We found that USP44 WT lowered the abundance of mono-, di-, and tri-ubiquitylated forms of H2A, whereas overexpression of USP44 CI led to a significant increase in higher-order ubiquitin chains on H2A (Fig. 3F). Together, these observations suggest that USP44 may regulate the ubiquitin-dependent DSB response by antagonizing ubiquitin chain formation on H2A.

USP44 Is Recruited to and Facilitates Reversal of RNF168-catalyzed Ubiquitylation Products—To further probe the possible involvement of USP44 in the ubiquitin-dependent signaling response to DSBs, we asked whether USP44 is physically recruited to the damaged chromatin. Strikingly, using cell lines stably expressing GFP-tagged USP44, we observed robust accumulation of catalytically inactive USP44 but not WT USP44 to microlaser-generated DNA damage tracks (Fig. 4A). Moreover, the accumulation of USP44 CI to laser sites was efficiently inhibited by knockdown of RNF8 or RNF168 (Fig. 4B), suggesting that USP44 is capable of both undergoing recruitment to and promoting hydrolysis of DSB-induced ubiquitylation products generated by RNF8/RNF168. In agreement with this notion, we found that formation of USP44 CI foci, which were not detectable in normal cells exposed to IR (data not shown), could be efficiently induced by stable overexpression of GFP-RNF168 (Fig. 4C). Although overexpressed USP44 readily displaced endogenous RNF168 from IRIF (Fig. 2B), presumably by counteracting RNF168-catalyzed H2A ubiquitylation in these regions, it failed to do so when RNF168 expression was elevated (Fig. 4C). Under these conditions, overexpressed USP44 was also unable to suppress 53BP1 recruitment to DSB sites (Fig. 4C). These and related findings support the notion that RNF168 and cellular activities, including USP44, that oppose DDR-associated H2A ubiquitylation engage in a constant tug-of-war and that the magnitude of downstream protein recruitment to IRIF is determined by the dynamic equilibrium between these activities. Notably, the recruitment of USP44 to RNF168-modified chromatin at DSB sites was unique among DUBs capable of counteracting RNF8/RNF8-dependent protein assembly at damaged chromatin (Fig. 1A) as inactive forms of neither USP3, USP29, nor OTUB1 formed detectable foci in IR-treated cells stably expressing GFP-RNF168 (Fig. 4D; data not shown). This further underscores a role of USP44 in reversing DSB-induced ubiquitylations generated by RNF8/RNF168.

The Extent of DSB-associated Histone Ubiquitylation Is Controlled by a Number of DUBs—The propagation of histone ubiquitylation and IRIF formation is determined by a dynamic equilibrium between opposing ubiquitin ligase and DUB activities. To address the extent to which endogenous USP44 regulates IRIF dynamics, we individually knocked down the expression of DUBs known to limit ubiquitylation and/or 53BP1 accumulation at DSB sites. To achieve unbiased evaluation of ubiquitin and 53BP1 foci formation capacity, we employed high content microscopy using the ScanR platform, an approach that we have previously used to quantify average foci intensities in large cohorts of cells (28). We focused on early time points (45 min) after DSB induction, where foci distribution is highly uniform, using low IR doses that do not exhaust the cellular capacity for 53BP1 recruitment to IRIF (20). In several independent experiments, we recorded a mild, but significant and highly reproducible, elevation in ubiquitin conjugates at DSB sites as well as 53BP1 foci intensity upon USP44 knockdown (Fig. 4, E and F). This effect could be observed with two inde-
pendent USP44 siRNAs and was comparable with the increase in FK2 and 53BP1 foci intensities seen after depletion of USP3 or OTUB1, which have previously been reported to counteract ubiquitin incorporation at sites of DNA damage (7, 21, 24) (Fig. 4, E and F). Simultaneous knockdown of two or more of these DUBs did not further enhance the intensity of FK2 and 53BP1 foci (data not shown), suggesting that additional factors and mechanisms also contribute to restricting ubiquitin-dependent protein recruitment to DSB-flanking chromatin.

**DISCUSSION**

The findings reported here establish USP44 as a DUB that is recruited to RNF8/RNF168-ubiquitylated chromatin surrounding DSBs and is capable of promoting hydrolysis of such...
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ubiquitin conjugates, thus negatively regulating protein recruitment to damaged chromatin. This adds USP44 to a growing list of factors that restrain DSB-associated, RNF8/RNF168-mediated chromatin ubiquitylation, either by facilitating the removal of such ubiquitylations or by interfering with their productive assembly or recognition. RNF168 has recently been shown to ubiquitylate the Lys-13 and Lys-15 residues in H2A-type histones rather than the canonical monoubiquitylation sites (6). Given the strong ability of USP44 but not a range of known H2A DUBs including BAP1 and USP16 to suppress RNF168-dependent protein recruitment to DSB sites, we suggest that USP44 may efficiently promote reversal of DSB-associated H2A Lys-13/Lys-15 ubiquitylation. Although we cannot rule out the possibility that USP44 reverses histone ubiquitylation indirectly via another factor, we consider it likely that USP44 directly catalyzes H2A deubiquitylation, particularly given that inactivation of the catalytic activity of USP44 efficiently traps it at RNF168-generated ubiquitylation products at DSB sites (Fig. 4). The cellular pathways restricting and reversing RNF8/RNF168-catalyzed ubiquitylation products are likely to display a considerable degree of functional redundancy, and so far no single DUB has been shown to be responsible for the bulk removal of these ubiquitylation products upon completion of DSB repair. The findings of this study recapitulate this notion in that knockdown of USP44, like that of OTUB1 or USP3, caused only a mild increase in accumulation of ubiquitin conjugates and 53BP1 at sites of DNA damage, whereas there was no apparent delay in the clearance of such foci after USP44, OTUB1, or USP3 depletion (data not shown). Thus, although overexpression of individual DUBs that counteract DSB-associated ubiquitylation of core histones can powerfully antagonize the DSB signaling response, knockdown of individual DUBs fulfilling this criterion only weakly interferes with its proper kinetics due to partial redundancy or overlapping functions with other DUBs. In such cases, which may be common in many aspects of cell biology, a DUB overexpression approach like the one presented in this study may provide a useful strategy to identify antagonizers of ubiquitin-dependent cellular responses that would not have been uncovered by siRNA-based screens. Our DUB expression plasmid inventory only encompassed some two-thirds of the human DUBs predicted to be catalytically active (29), and so it remains possible that yet additional DUBs may be capable of limiting protein recruitment to DSB sites when overproduced. Furthermore, not all DUBs may be inherently active upon mere overexpression. For instance, the activities of USP1, USP12, and USP46 have been shown to critically require the presence of their partner subunit UAF1 (41), and several other DUBs require further posttranslational modification for full activation.

Our finding that USP44 promotes histone H2A deubiquitylation is well aligned with a recent study showing that USP44 deubiquitylates histone H2B to regulate gene expression during stem cell differentiation (36). Other recent studies point to a role for USP44 as a tumor suppressor that counteracts the activity of the anaphase-promoting complex and regulates centrosome positioning during mitosis (38, 42). It is possible that the mitotic defects and aneuploidy observed for USP44-deficient cells could be at least partially attributed to the activity of USP44 toward core histones. In light of our findings that USP44 can also modulate the DNA damage response, it is possible that the tumor suppressor and genome stability maintenance function of USP44 may not solely reflect its involvement in regulation of mitosis.

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