UBR5-mediated ubiquitination of ATMIN is required for ionizing radiation-induced ATM signaling and function

Tianyi Zhang[a,1], Janet Cronshaw[a], Nnennaya Kanu[a,2], Ambrosius P. Snijders[b], and Axel Behrens[a,c,3]

[a]Mammalian Genetics Laboratory, Cancer Research UK London Research Institute, London WC2A 3LY, United Kingdom; [b]Protein Analysis and Proteomics Laboratory, Clare Hall Laboratories, Cancer Research UK London Research Institute, South Mimms EN6 3LD, Hertfordshire, United Kingdom; and [c]School of Medicine, Guy's Campus, King's College London, London SE1 1UL, United Kingdom

Edited by Stephen J. Elledge, Harvard Medical School, Boston, MA, and approved July 3, 2014 (received for review January 6, 2014)

The Mre11/Rad50/NBS1 (MRN) protein complex and ATMIN protein mediate ATM kinase signaling in response to ionizing radiation (IR) and chromatin changes, respectively. NBS1 and ATMIN directly compete for ATM binding, but the molecular mechanism favoring either NBS1 or ATMIN in response to specific stimuli is enigmatic. Here, we identify the E3 ubiquitin ligase UBR5 as a key component of ATM activation in response to IR. UBR5 interacts with ATMIN and catalyzes ubiquitination of ATMIN at lysine 238 in an IR-stimulated manner, which decreases ATMIN interaction with ATM and promotes MRN-mediated signaling. We show that UBR5 deficiency, or mutation of ATMIN lysine 238, prevents ATMIN dissociation from ATM and inhibits ATM and NBS1 foci formation after IR, thereby impairing checkpoint activation and increasing radiosensitivity. Thus, UBR5-mediated ATMIN ubiquitination is a vital event for ATM pathway selection and activation in response to DNA damage.

ATM kinase is part of the phosphatidylinositol 3-kinase–related kinase (PIKK) family that activates cell-cycle checkpoints and promotes DNA repair in response to DNA damage or replication blocks (1). Mutation of *ATM* causes the genomic instability syndrome ataxia telangiectasia, characterized by cerebellar degeneration, immunodeficiency, and increased tumor incidence (2).

In response to DNA double-strand breaks (DSBs), inactive ATM homodimers dissociate and the kinase is activated (3), phosphorylating other ATM molecules, as well as numerous substrates including structural maintenance of chromosomes protein 1 (SMC1) and p53, at serine or threonine residues followed by glutamine (the "SQ/TQ" motif) (4, 5). ATM is activated at DSB sites via the Mre11/Rad50/NBS1 (MRN) complex, which is required for ATM activation and recruitment into nuclear foci, and MRN interacts with ATM mainly via its NBS1 subunit (6, 7). A short C-terminal motif in NBS1 principally contributes to ATM binding (8), and the interaction is also strengthened by ubiquitination of NBS1 (9).

ATM not only is central to the DSB response but also responds to many other cellular stresses, such as UV damage and hypotonic stress (1, 3). In contrast to its role at DSB sites, NBS1 is not required for ATM activation by these stimuli (10, 11); instead, ATM is activated via interaction with its cofactor ATMIN (12). Accordingly, ATMIN colocalizes with phosphorylated ATM in basal conditions and after hypotonic stress, but not after ionizing radiation (IR). The mechanism of the switch between these different signaling conditions is incompletely understood. Our previous work has indicated that competitive interaction of either NBS1 or ATMIN with ATM is part of this switch and that overexpression of ATMIN can inhibit NBS1-mediated ATM activation (11). However, the signaling mechanism(s) that favor interaction with one protein over the other in different conditions are unknown.

Ubiquitin protein ligase E3 component n-recognin 5 (UBR5) is a very large protein of 2,799 amino acids (309 kDa) belonging to the HECT family of E3 ubiquitin ligases. UBR5 also has E3-independent roles as a transcriptional cofactor for the progesterone receptor (13) and interacts with other proteins such as p53 (14). UBR5 has been shown to mediate degradation of several proteins, including katanin p60, beta-catenin, TOPBP1, and TERT protein, the catalytic subunit of telomerase (15–18). UBR5 has also been implicated in the DNA damage response: it interacts with Chk2 and is necessary for Chk2 phosphorylation and cell-cycle arrest in response to IR (19, 20). More recently, UBR5, together with another E3 ubiquitin ligase, Trip12, has been shown to restrict histone ubiquitination and p53-binding protein 1 (53BP1) focus formation at DNA damage sites (21).

Here, we further elucidate the role of UBR5 in the cellular response to DSBs and identify ATMIN as a crucial substrate of ubiquitination by UBR5 for the protection of genome stability.

Results

ATMIN Interacts with the Ubiquitin Ligase UBR5. We previously discovered that levels of the ATM interactor ATMIN influence the output of ATM signaling following IR, likely by competing with the MRN subunit NBS1 for ATM binding (11). Based on this result, the interaction of ATMIN with ATM must be carefully controlled to enable appropriate ATM activation. To investigate potential regulatory mechanisms affecting ATMIN's interaction with ATM, we have used mass spectrometry to identify proteins physically interacting with ATMIN. One of these proteins was the HECT family ubiquitin ligase UBR5, also

Significance

The checkpoint kinase ATM directs the cellular response to ionizing radiation (IR) by localizing to DNA damage sites and actively phosphorylating proteins involved in repair and survival. ATM is recruited and activated at damage sites via an interaction with the Mre11/Rad50/NBS1 (MRN) complex. We have previously shown that an alternative ATM binding partner, ATMIN, competitively inhibits this interaction, suggesting that there must be a mechanism preventing ATMIN from disrupting ATM signaling in IR conditions. Here, we show that ATMIN is ubiquitinated by the E3 ligase UBR5, a modification that is stimulated by IR and favors its dissociation from ATM, freeing ATM to interact with NBS1. This mechanism allows efficient ATM activation at damage sites and promotes cell survival after irradiation.

Author contributions: T.Z., N.K., and A.B. designed research; T.Z., J.C., N.K., and A.P.S. performed research; T.Z., N.K., A.P.S., and A.B. analyzed data; and T.Z. and A.B. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]Present address: Institute of Medical Biology, 06-06 Immunos, Singapore 138648.

[2]Present address: Translational Cancer Therapeutics Laboratory, University College London Cancer Institute, London WC1E 6BT, United Kingdom.

[3]To whom correspondence should be addressed. Email: axel.behrens@cancer.org.uk.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1400230111/-/DCSupplemental.

known as hyperplastic discs protein homolog (HYD) and E3 ligase identified by differential display (EDD) (14, 18, 19, 21, 22) (Fig. S1*A*). Immunoprecipitated endogenous ATMIN was able to pull down endogenous UBR5 (Fig. 1*A*), and, conversely, tagged UBR5 coimmunoprecipitated GFP-ATMIN, in both unstimulated and IR conditions (Fig. 1*B*).

UBR5 Ubiquitinates ATMIN in an IR-Stimulated Manner. Because UBR5 is a ubiquitin ligase, we next determined whether UBR5 is able to ubiquitinate ATMIN. Overexpression of Flag-tagged UBR5 induced ubiquitination of Flag-tagged ATMIN in untreated cells (Fig. 1*C*, lanes 2 and 3). Interestingly, this modification was greatly stimulated when cells were treated with IR (Fig. 1*C*, lanes 3 and 5). The interaction between UBR5 and ATMIN was not dependent on UBR5 E3 ligase activity (Fig. S1*B*). In IR conditions, depletion of UBR5 using siRNA strongly reduced ATMIN ubiquitination (Fig. 1*D*). Also, ubiquitination of endogenous ATMIN protein could be detected in IR-treated cells (Fig. 1*E*). In contrast, exposure of cells to osmotic stress did not stimulate ubiquitination of ATMIN by UBR5 (Fig. S1*C*). These results suggest that UBR5 physically interacts with and ubiquitinates ATMIN and that this modification is stimulated by IR treatment.

ATMIN Is Ubiquitinated at Lysine 238. To determine where on the protein ATMIN is ubiquitinated, we first used several constructs encoding truncations of the ATMIN protein. Most regions of ATMIN, including the C terminus (amino acids 625–818) that is sufficient for ATM interaction (11), showed no ubiquitination following UBR5 overexpression and ubiquitin pulldown (Fig. 2*A*, *Lower*). In contrast, ubiquitination of the N-terminal portion of ATMIN (amino acids 1–354) was detected in whole-cell extract ("input") and more strongly in the immunoprecipitate (Fig. 2*A*, *Upper*). In agreement with the findings for the full-length protein, the N-terminal portion of ATMIN was ubiquitinated in an IR-stimulated manner (Fig. 2*B*). Both basal and IR-induced ATMIN ubiquitination were dependent on catalytically active UBR5 because an E3 ligase defective HECT domain mutant (C2768A) (13) was unable to support ATMIN ubiquitination (Fig. 2*B*). Notably, ATMIN did not appear to be destabilized either by IR treatment or by overexpression of UBR5, suggesting that the ubiquitin modification is not linked to proteasomal degradation of ATMIN.

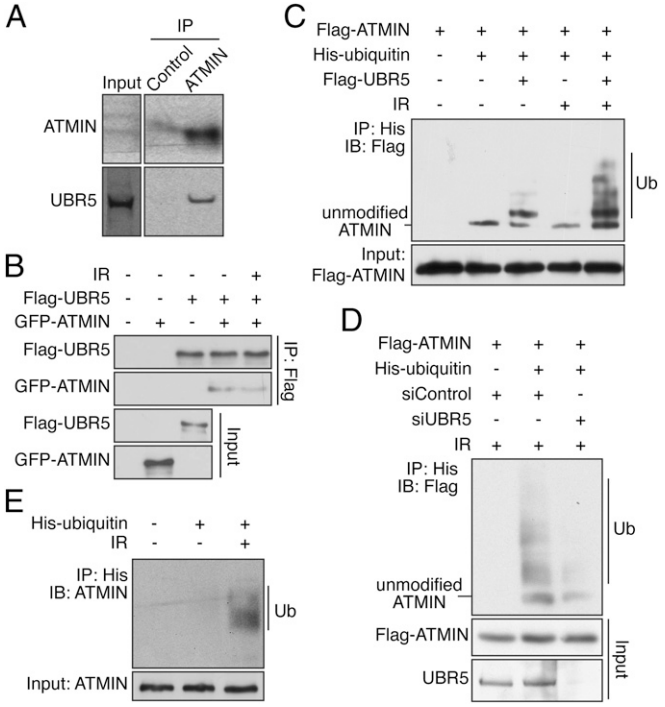

Fig. 1. UBR5 interacts with ATMIN and mediates ATMIN ubiquitination. (*A*) Coimmunoprecipitation (CoIP) of endogenous ATMIN and endogenous UBR5 from HeLa cells under basal conditions. (*B*) CoIP of GFP-tagged ATMIN and Flag-tagged UBR5 from a mixture of 293T whole-cell lysates expressing the individual constructs or vector control. Cells were treated with proteasome inhibitor for 4 h before harvest. (*C*) Ubiquitin pulldown from 293 cells expressing Flag-ATMIN and Flag-UBR5. Lysates were previously equilibrated for Flag-ATMIN input levels. (*D*) Ubiquitin pulldown from 293T cells transfected with siRNA against UBR5 or siControl followed by Flag-ATMIN and His-ubiquitin constructs. (*E*) Ubiquitin pulldown from 293T cells, showing ubiquitination of endogenous ATMIN. IP, immunoprecipitation or Ni-NTA pulldown; IB, immunoblot.

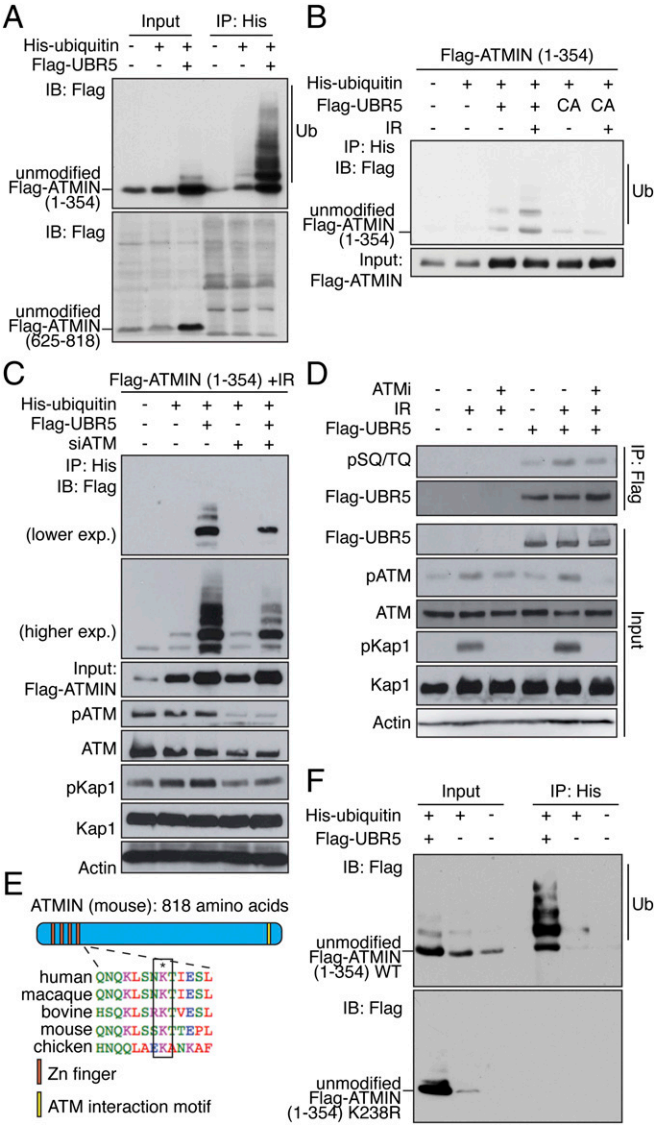

Fig. 2. UBR5 ubiquitinates ATMIN N terminus at K238. (*A*) Ubiquitin pulldown from 293T cells transfected with either Flag-ATMIN N terminus (residues 1–354) or C terminus (residues 625–818). (*B*) Ubiquitin pulldown from 293T cells transfected with either wild-type Flag-UBR5 (+) or C2768A (CA) E3 ligase defective mutant Flag-UBR5. (*C*) Ubiquitin pulldown (*Upper*) and Western blots of whole-cell lysates (*Lower*) from 293T cells transfected with siATM or siControl (–) for 72 h, followed by Flag-ATMIN (1–354) constructs. (*D*) IP of Flag-tagged UBR5 from 293T cells treated with ATM inhibitor (ATMi) and/or IR as indicated. (*E*) Scheme of ATMIN domains and conserved ubiquitination site. (*F*) Ubiquitin pulldown from 293T cells transfected with either wild-type Flag-ATMIN(1-354) or K238R mutant ATMIN(1-354).

IR treatment triggers activation of DNA damage kinases of the PIKK family, including ATM, ATR, and DNA-dependent protein kinase (DNA-PK). Because ATMIN ubiquitination is stimulated by IR, we determined whether any of these kinases is responsible for this apparent increase in ATMIN ubiquitination. When cells were treated with ATM inhibitor, or caffeine, which inhibits all PIKK family kinases, IR-induced phosphorylation of ATM substrates was inhibited, and likewise, ubiquitination of ATMIN was reduced, whereas DNA-PK inhibitor had little effect (Fig. S2*A*). Depletion of ATM by siRNA also reduced the UBR5-mediated ubiquitination of ATMIN (Fig. 2*C*). UBR5 is known to be phosphorylated on several SQ/TQ sites following DNA damage (23, 24), raising the possibility that it is directly modified by ATM in response to IR. Probing immunoprecipitated UBR5 with a pSQ/TQ antibody in the absence or presence of ATM inhibitor showed that IR-induced phosphorylation of UBR5 at these site(s) was dependent on ATM activity (Fig. 2*D*), consistent with direct activation of UBR5 by ATM.

ATMIN consists of 818 amino acids in the mouse, and the N-terminal region alone contains 25 lysine residues. To determine which among these lysines are ubiquitination sites, we performed mass spectrometry of ATMIN(1–354) (Fig. S2 *B* and *C*). This analysis revealed a ubiquitin modification at a single site, lysine 238. Notably, this site is conserved among several other species, including humans (Fig. 2*E*). When lysine 238 was mutated to arginine (ATMIN-K238R), UBR5 was no longer able to modify the protein (Fig. 2*F*). Thus, UBR5 ubiquitinates ATMIN on lysine 238.

ATMIN Ubiquitination at Lysine 238 Is Required for IR-Induced ATM Signaling. The above data suggest that ATM phosphorylates and activates UBR5 in response to IR, resulting in ubiquitination of ATMIN. To determine whether this activity in turn affects ATM signaling, we first depleted UBR5 using siRNA and examined IR-induced phosphorylation of ATM substrates and formation of repair foci. IR-induced phosphorylation of the ATM substrates SMC1 and KRAB-associated protein 1 (Kap1) was markedly reduced in UBR5-depleted cells (Fig. 3*A*). To analyze the effect of UBR5 depletion in individual cells, we stained IR-treated cells with anti-53BP1 and pATM. Although 53BP1 and pATM formed foci as expected in control cells, foci were reduced in UBR5-depleted cells (Fig. 3 *B* and *C*).

Our previous data showed that ATMIN interacts with ATM, but this interaction is reduced following IR treatment (12). To examine whether UBR5-dependent ubiquitination at K238 affects the interaction of ATMIN with ATM, we used tagged wild-type ATMIN, or the ATMIN-K238R mutant, to immunoprecipitate ATM. Although the interaction between wild-type ATMIN and ATM was only weakly detectable, ATMIN-K238R was able to pull down ATM much more efficiently than the wild-type protein (Fig. 3*D*, compare lanes 2 and 4), suggesting that modification of K238 destabilizes the ATMIN-ATM interaction. Furthermore, depleting UBR5 also increased the efficiency of ATMIN-ATM binding, phenocopying the K238R mutation (Fig. 3*D*, compare lanes 2 and 3). Notably, depleting UBR5 in the presence of mutant ATMIN did not further increase the ATMIN-ATM interaction (Fig. 3*D*, lane 5). UBR5 depletion also increased endogenous ATMIN-ATM binding (Fig. 3*E*, compare lanes 3 and 5). These data suggest that, when K238 is mutated or UBR5 is depleted, ATMIN is unable to be ubiquitinated and ATMIN interaction with ATM is increased, thereby disrupting IR-induced ATM signaling. In line with the above data indicating that ATM is required for UBR5 activation, inhibition of ATM activity prevented its dissociation from ATMIN in IR conditions (Fig. 3*E*). This result suggests a positive feedback mechanism whereby initial ATM activation stimulated by IR activates UBR5, which ubiquitinates ATMIN and promotes its dissociation from ATM, allowing further ATM activation. A time course following IR stimulation supported this notion, with ATM autophosphorylation appearing within 5 min, but ATMIN ubiquitination and phosphorylation of the ATM substrate Kap1 not peaking until 15–30 min post IR (Fig. S3*A*).

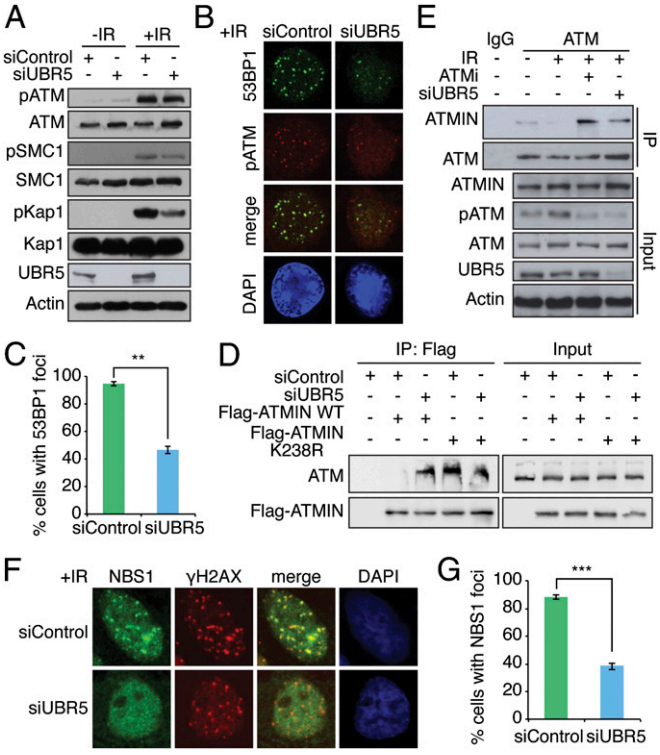

Fig. 3. Loss of UBR5 increases ATM-ATMIN interaction and impairs ATM signaling after IR. (*A*) Western blots of whole-cell lysates from 293T cells transfected with siUBR5 or siControl. (*B*) Knockdown of UBR5 impairs 53BP1 and pATM foci formation in 293A cells after IR. (*C*) Quantification of 53BP1 positive cells (with at least six distinct foci). (*D*) CoIP of ATM and Flag-ATMIN in 293T cells depleted for UBR5 and/or expressing K238R mutant ATMIN. (*E*) CoIP of endogenous ATMIN with endogenous ATM. (*F*) NBS1 and γH2AX foci formation 30 min after IR. (*G*) Quantification of NBS1-positive cells (with at least five distinct foci). Error bars represent SEM (**$P < 0.01$, ***$P < 0.005$).

We previously found that ATMIN overexpression disrupts IR-induced ATM signaling by increasing the amount of ATM bound to ATMIN and consequently reducing ATM's capacity to interact with NBS1 (11). We therefore asked whether UBR5 depletion affects the NBS1 response to IR. In control cells, NBS1 formed IR-induced foci, but after UBR5 depletion, the number of cells with NBS1 foci was reduced by over 50% (Fig. 3 *F* and *G*). This result suggests that UBR5 is required for efficient NBS1 foci formation. Depletion of UBR5, ATM, or ATMIN did not affect NBS1 protein levels, suggesting that UBR5 specifically affects NBS1 focus formation (Fig. S3*B*). Although NBS1 recruitment to DSB sites is independent of ATM, phosphorylation by ATM is required for NBS1 accumulation into foci (25–27). UBR5 depletion could therefore impair NBS1 foci formation by reducing ATM–NBS1 interaction and thus ATM signaling at DSB sites. Consistent with this idea, γH2AX levels were reduced, and foci also appeared more diffuse in UBR5-depleted cells, consistent with defective amplification and stabilization of H2AX phosphorylation by ATM (Fig. 3*F* and Fig. S3*B*). Taken together, these data indicate that preventing ATMIN dissociation from ATM by UBR5 depletion leads to impaired MRN-dependent ATM signaling.

ATMIN Is the Essential Substrate of UBR5 Involved in IR-Induced ATM Signaling. To investigate the importance of ATMIN as a UBR5 substrate in IR-induced ATM signaling, we made use of ATMIN-null mouse embryonic fibroblasts (MEFs). In agreement with the previous results, IR-induced phosphorylation of SMC1 and Kap1 was reduced by depletion of UBR5 in wild-type MEFs (Fig. 4*A*, lanes 5 and 6). Importantly, in MEFs lacking both ATMIN and

CELL BIOLOGY

UBR5, phosphorylation of Kap1 was similar to that in wild-type MEFs, indicating that low levels of UBR5 do not inhibit ATM signaling in the absence of ATMIN (Fig. 4*A*, lanes 7 and 8). Thus, ATMIN is a functionally relevant substrate of UBR5 in IR-induced ATM signaling.

Interestingly, UBR5 protein was barely detectable in ATMIN-null cells following IR treatment, suggesting that ATMIN protein is required to maintain UBR5 protein levels after IR (Fig. 4*A*, lanes 7 and 8). Conversely, ATMIN overexpression resulted in significantly increased protein levels of UBR5 (Fig. S3*C*). Proteasome inhibition also resulted in substantially higher UBR5 protein levels (Fig. S3*D*), indicating that UBR5, similar to other E3 ubiquitin ligases, may undergo degradative autoubiquitination. In accordance with the previous data showing that UBR5 E3 ligase activity is stimulated by ATM in IR conditions, inhibition of ATM prevented the IR-induced depletion of UBR5 in ATMIN-null cells (Fig. S3*E*). Thus, it is possible that binding to ATMIN prevents UBR5 autoubiquitination.

Consistent with our previous study (11), overexpression of ATMIN impaired IR-induced ATM signaling (Fig. 4 *B–F*). Importantly, however, UBR5 overexpression was able to rescue IR-induced phosphorylation of ATM, SMC1, and Kap1 and 53BP1 foci formation in ATMIN-overexpressing cells (Fig. 4 *B–D*). ATMIN overexpression reduced NBS1 accumulation at DSBs marked by γH2AX, and this impairment was also partially rescued by co-overexpression of UBR5 (Fig. 4 *E* and *F*). These results are consistent with a model where UBR5 promotes IR-induced signaling by antagonizing ATMIN interaction with ATM. In line with this model, UBR5 overexpression had little effect on ATM signaling responses in the absence of ATMIN overexpression (Fig. 4 *B–F*). The observation that overexpression of ATMIN phenocopies depletion of UBR5 also supports the hypothesis that UBR5 restricts ATMIN's ability to compete with NBS1 for ATM.

ATMIN Lysine 238 Is Required for ATM Signaling and Function. ATM function is required for cell-cycle checkpoints, including the G2/M checkpoint in response to IR. To assess the role of ATMIN K238 ubiquitination in the ATM-dependent DNA damage response, we measured IR-induced G2/M checkpoint activation in 293T cells expressing either wild-type ATMIN or the ATMIN-K238R mutant. As expected, irradiation reduced the number of control cells entering mitosis, indicating an active G2/M checkpoint (Fig. 5*A* and Fig. S4*A*). In contrast, in cells expressing ATMIN-K238R, there was no reduction in mitotic index after low-dose irradiation, indicating that the IR checkpoint is not functional (Fig. 5*A* and Fig. S4*A*).

To further evaluate the function of ATMIN K238 ubiquitination, we reconstituted ATMIN-null MEFs with either wild-type ATMIN or ATMIN-K238R (Fig. S4*B*). ATMIN mRNA and protein levels in reconstituted cells were moderately increased compared with endogenous ATMIN, but ectopically expressed wild-type ATMIN and ATMIN-K238R protein levels were comparable (Fig. S4 *C* and *D*). $ATMIN^{f/f}$ MEFs reconstituted with control empty vector showed strong 53BP1 foci in response to IR (Fig. 5*B*). Reconstitution of wild-type ATMIN in $ATMIN^{\Delta/\Delta}$ cells resulted in mildly reduced IR-induced foci formation, as expected by the modestly increased ATMIN levels (Fig. 5 *B* and *C*). However, ATMIN-K238R reconstitution strongly reduced 53BP1 foci formation in response to IR (Fig. 5 *B* and *C*). In line with these results, phosphorylation of the ATM substrates SMC1 and Kap1, as well as ATM itself, was strongly reduced in $ATMIN^{\Delta/\Delta}$ cells complemented with ATMIN-K238R compared with $ATMIN^{\Delta/\Delta}$ plus wtATMIN cells (Fig. 5*D*),

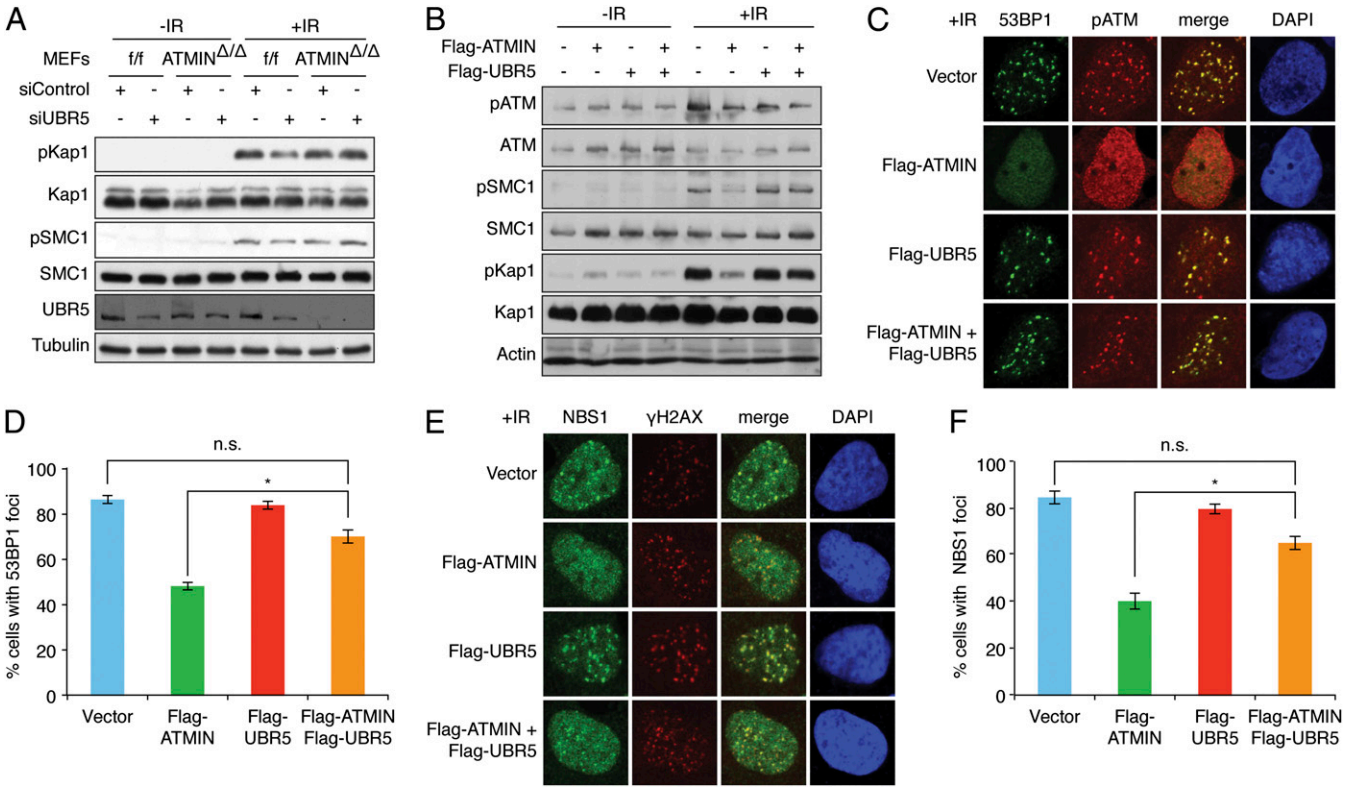

Fig. 4. IR signaling impaired by ATMIN is rescued by expression of UBR5. (*A*) Western blots of whole-cell lysates from wild-type or *ATMINΔ/Δ* MEFs (11) transfected with siRNA against UBR5 or control siRNA. (*B*) Western blots of whole-cell lysates from 293T cells transfected with Flag-ATMIN, Flag-UBR5, or both. (*C*) Immunofluorescence staining for 53BP1 and pATM foci in 293A cells transfected with Flag-ATMIN, Flag-UBR5, or both and fixed 30 min after IR. (*D*) Quantification of 53BP1-positive cells (with at least six distinct foci). (*E*) Immunofluorescence staining for NBS1 and γH2AX foci in 293A cells transfected with Flag-ATMIN, Flag-UBR5, or both and fixed 30 min after IR. (*F*) Quantification of cells positive for NBS1 foci (with at least five distinct foci). Error bars represent SEM (*$P < 0.05$; n.s., not significant).

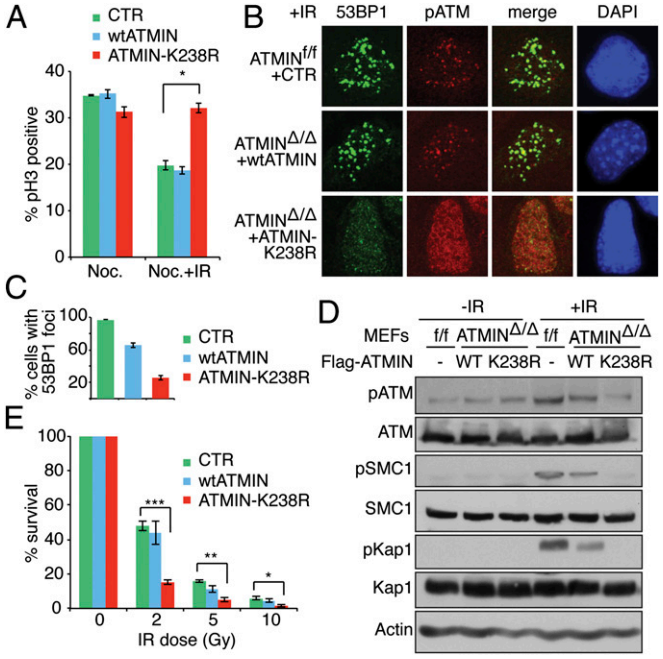

Fig. 5. ATMIN ubiquitination is required for ATM signaling and checkpoint function post IR. (*A*) G2/M nocodazole trap and quantification of mitotic index in 293T cells transfected with vector (CTR), Flag-ATMIN wild type, or Flag-ATMIN K238R, measured by FACS of phospho-histone 3 (pH3)-positive cells. (*B*) 53BP1 and pATM immunofluorescence staining of *ATMIN*$^{f/f}$ MEFs transfected with empty vector (ATMIN$^{f/f}$+CTR) or *ATMIN*$^{\Delta/\Delta}$ MEFs reconstituted with either wild-type (+wtATMIN) or K238R mutant Flag-ATMIN (+ATMIN-K238R). (*C*) Quantification of 53BP1-positive cells (with at least six distinct foci) from the experiment in *B*. (*D*) Western blots of whole-cell lysates from MEFs treated as in *B*. (*E*) Radiosensitivity assay of reconstituted MEFs as in *B*, showing percentage of surviving colonies 7 d after IR. Error bars represent SEM (***$P < 0.005$, **$P < 0.01$, *$P < 0.05$).

indicating that modification of ATMIN at K238 is required for robust ATM signaling after IR.

A defect in ATM signaling after IR, such as in ATM-deficient cells, would be expected to induce radiosensitivity. We therefore quantified cell survival following IR treatment in the ATMIN-reconstituted MEFs. ATMIN-K238R–reconstituted cells showed significantly lower survival after IR treatment compared with ATMIN wild-type MEFs (Fig. 5*E*). Thus, modification of ATMIN at K238 is required for ATM-mediated radioresistance as well as activation of checkpoint signaling.

Discussion

The MRN complex is required for ATM activation by DSBs (7, 28) whereas ATM signaling triggered by changes in chromatin structure requires ATMIN (12). NBS1 and ATMIN proteins compete for ATM binding, and this mechanism underlies ATM pathway choice and function (11). However, the mechanism that instructs ATM to enter either the MRN-dependent or the ATMIN-dependent signaling pathway was enigmatic. Here, we shed light on this decision, by identifying UBR5 ubiquitination of ATMIN as a key step in the activation of ATM signaling by IR.

ATMIN Ubiquitination Is Required for IR-Induced ATM Signaling. Ubiquitination has important functions in many aspects of biological activity. Although ubiquitination was originally thought only to target proteins for degradation, there are many additional roles of ubiquitination in nonproteolytic functions, including DNA repair (29). In this study, we show that ATMIN undergoes ubiquitination upon IR treatment and that this modification does not trigger ATMIN degradation. Instead, ATMIN ubiquitination decreases the interaction of ATMIN with ATM, thereby facilitating ATM function at DSBs. Notably, overexpression of the ATMIN ubiquitination-deficient mutant (K238R) strongly inhibits ATM activation upon IR treatment (Fig. 5). The site of ATMIN ubiquitination is separated by about 500 amino acids from the ATM interaction motif, a main point of ATMIN/ATM interaction (11, 12). ATMIN ubiquitination may thus impair ATM binding by steric interference or could induce an allosteric change in ATMIN that decreases affinity for ATM. It is noteworthy that, in ATMIN-deficient cells, UBR5 levels greatly decrease in response to IR (Fig. 4*A* and Fig. S3*E*), and that ATMIN overexpression significantly increases UBR5 protein levels (Fig. S3*C*). Many E3 ubiquitin ligases regulate their protein levels by autoubiquitination, and the dramatic increase in UBR5 protein following proteasome inhibition (Fig. S3*D*) suggests that this is also the case for UBR5. Interestingly, the increase in UBR5 levels with proteasome inhibitor occurs only when ATMIN is absent (Fig. S3*E*) or when UBR5 is overexpressed (Fig. S3*D*). It is thus possible that, if the preferred substrate ATMIN is not available, IR-induced UBR5 activity may result in autoubiquitination and degradation.

UBR5 Is Stimulated by IR. In response to IR, UBR5 modification of ATMIN is stimulated, resulting in increased ATMIN ubiquitination. Although it is possible that this increase is due to increased availability of the K238 site, or other changes, our data indicate that modification of UBR5 itself may also increase its enzymatic activity. UBR5 is a heavily phosphorylated protein, and many phosphorylation sites on UBR5 have been reported in the literature, mostly identified by large-scale proteomics studies (23, 24). Consistent with these studies, we found that UBR5 was phosphorylated on SQ/TQ site(s), predicted phosphorylation sites of ATM/ATR kinases, in response to IR (Fig. 2*D*). Our finding that ATM inhibition or depletion reduced UBR5 phosphorylation and ATMIN ubiquitination (Fig. 2 *C* and *D*) supports the notion that ATM phosphorylates UBR5 in IR conditions. It is therefore conceivable that increased activity of UBR5 after IR is at least in part mediated by ATM. We speculate that the earliest stages of IR-induced ATM activation do not depend on UBR5, but that this initial ATM activity is

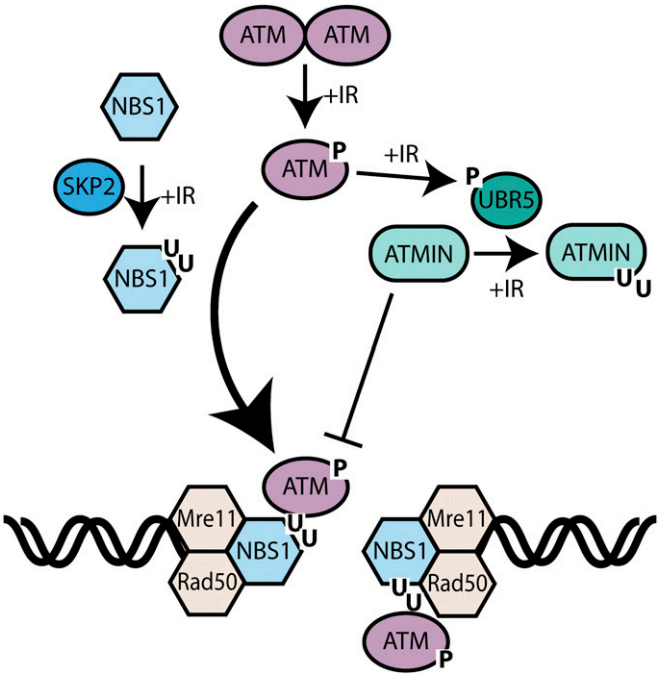

Fig. 6. Simplified model showing IR-induced posttranslational modifications of NBS1, ATM, UBR5, and ATMIN enabling assembly of the MRN complex and active ATM at DSBs.

amplified by a positive feedback loop involving UBR5. Our time-course data (Fig. S3*A*) support this idea. According to this model, low levels of ATM signaling shortly after IR treatment increase UBR5 activity, and UBR5 in turn catalyzes ATMIN ubiquitination, impairs ATM/ATMIN association, and subsequently results in increased binding of ATM to the MRN complex, promoting and maintaining further ATM signaling. Such positive feedback systems of posttranslational modification are already known to act in DNA repair: for example, in spreading of γH2AX from a double-strand break site (30). At present, it is unclear why osmotic stress, which triggers ATM activation, does not result in the activation of UBR5 and ubiquitination of ATMIN (Fig. S1*C*). It is possible that these different stimuli activate different subcellular pools of ATM or that UBR5 requires a second, ATM-independent stimulus following IR for full activation. These additional controls would be necessary to ensure that ATMIN does not dissociate from ATM in conditions where it is needed for signaling.

Ubiquitination Mediates the Switch from ATMIN- to MRN-Dependent ATM Signaling. The MRN complex is responsible for the initial recognition of DSBs upon genotoxic stress and recruits ATM to DNA damage foci for its subsequent activation (6). NBS1 is a key component of the MRN complex, central to the ability of the MRN complex to activate ATM (28). Interestingly, NBS1 as well as ATMIN is modified by ubiquitination upon IR treatment. Recent work showed that S-phase kinase-associated protein 2 (Skp2) E3 ligase is a critical regulator required for the recruitment of ATM by the MRN complex and for subsequent ATM activation in response to DSBs. Skp2 triggers K63-linked ubiquitination of NBS1, which increases NBS1 interaction with ATM, in turn facilitating activation and recruitment of ATM to DNA damage foci (9).

Although NBS1 ubiquitination increases its interaction with ATM, ATMIN ubiquitination decreases its affinity for ATM, thereby mediating the switch from ATMIN- to MRN-dependent ATM signaling in response to IR. Thus, ionizing radiation controls ATM signaling by simultaneously facilitating MRN-dependent ATM signaling and antagonizing ATMIN-dependent ATM signaling. In addition, the competitive relationship between ATMIN and NBS1 implies that reduced interaction with ATMIN also contributes to this switch by making more active monomeric ATM available for interaction with MRN (11). Together with these studies, our results suggest a model in which IR-induced ubiquitination of two key molecules that determine ATM pathway choice, ATMIN and NBS1, is an essential mechanism promoting ATM signaling at DSBs (Fig. 6).

Materials and Methods

Cell Treatments. IR dose was 2 Gy unless otherwise stated. ATM inhibitor (118500; EMD Millipore) was used at 10 μM for 30 min, before IR treatment. siRNA was transfected 24 h before any overexpression constructs, and cells were treated with IR after a further 48 h. For MEF reconstitution, *ATMIN$^{f/f}$; Rosa-creERT* MEFs were immortalized with SV40 large T antigen and then retrovirally infected with empty vector, wild-type Flag-ATMIN, or K238R Flag-ATMIN for 3 d. MEFs were FACS sorted for GFP and subsequently deleted for endogenous *ATMIN* in culture by addition of 4-hydroxytamoxifen (Sigma).

Immunoprecipitation and Ubiquitin Pulldown. The 293T cells were transfected and irradiated before lysis for 30 min at 4 °C in 500 μL of immunoprecipitation (IP) buffer. After centrifugation, supernatants were incubated overnight at 4 °C with FlagM2 agarose beads. For in vivo ubiquitin assay, HEK293 cells were transfected with His-ubiquitin, Flag-tagged ATMIN, and Flag-tagged UBR5 using a calcium phosphate protocol (Profection). After 48 h, cells were harvested, and the amount of Flag-ATMIN in lysates was equilibrated. After lysate incubation with Ni-NTA beads (Qiagen) at 4 °C overnight, beads were washed, and the IP mixture was boiled in 2× SDS loading buffer in the presence of 200 mM imidazole. The eluted proteins were analyzed by Western blot for ATMIN ubiquitination by probing with Flag-HRP (Sigma).

G2/M Checkpoint Assay and Radiosensitivity Assay. Cells were irradiated 24 h after transfection with a Cs137 Gamma Irradiator at 2.1 Gy/min. Immediately, 100 nM nocodazole was added, and cells were fixed after 18 h in 70% (vol/vol) ethanol. Fixed cells were stained with phospho-Histone 3 antibody and analyzed using a BD Biosciences FACScan. For radiosensitivity assay, MEFs were irradiated, trypsinized, and replated in triplicate. After 7 d, the number of colonies on each plate was manually counted.

For details of cell culture, buffers, primer sequences, mass spectrometry, immunofluorescence, antibodies, and expression plasmids, please see *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank P. Meier for critical reading of the manuscript and the Mammalian Genetics Laboratory for input and discussions. Support was given from the Protein Analysis and Proteomics Unit, the Light Microscopy Unit, and the FACS Laboratory at the London Research Institute. T.Z. was supported by an Agency for Science, Technology and Research (A*STAR) National Science Scholarship-PhD scholarship (Singapore). This work was supported by European Research Council Grant 281661 ATMINDDR (to A.B.). The London Research Institute is funded by Cancer Research UK.

1. Shiloh Y, Ziv Y (2013) The ATM protein kinase: Regulating the cellular response to genotoxic stress, and more. *Nat Rev Mol Cell Biol* 14(4):197–210.
2. McKinnon PJ (2004) ATM and ataxia telangiectasia. *EMBO Rep* 5(8):772–776.
3. Bakkenist CJ, Kastan MB (2003) DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. *Nature* 421(6922):499–506.
4. Shiloh Y (2003) ATM and related protein kinases: Safeguarding genome integrity. *Nat Rev Cancer* 3(3):155–168.
5. Kim ST, Lim DS, Canman CE, Kastan MB (1999) Substrate specificities and identification of putative substrates of ATM kinase family members. *J Biol Chem* 274(53):37538–37543.
6. Lee JH, Paull TT (2005) ATM activation by DNA double-strand breaks through the Mre11-Rad50-Nbs1 complex. *Science* 308(5721):551–554.
7. Uziel T, et al. (2003) Requirement of the MRN complex for ATM activation by DNA damage. *EMBO J* 22(20):5612–5621.
8. Falck J, Coates J, Jackson SP (2005) Conserved modes of recruitment of ATM, ATR and DNA-PKcs to sites of DNA damage. *Nature* 434(7033):605–611.
9. Wu J, et al. (2012) Skp2 E3 ligase integrates ATM activation and homologous recombination repair by ubiquitinating NBS1. *Mol Cell* 46(3):351–361.
10. Difilippantonio S, et al. (2005) Role of Nbs1 in the activation of the Atm kinase revealed in humanized mouse models. *Nat Cell Biol* 7(7):675–685.
11. Zhang T, et al. (2012) Competition between NBS1 and ATMIN controls ATM signaling pathway choice. *Cell Reports* 2(6):1498–1504.
12. Kanu N, Behrens A (2007) ATMIN defines an NBS1-independent pathway of ATM signalling. *EMBO J* 26(12):2933–2941.
13. Henderson MJ, et al. (2002) EDD, the human hyperplastic discs protein, has a role in progesterone receptor coactivation and potential involvement in DNA damage response. *J Biol Chem* 277(29):26468–26478.
14. Ling S, Lin WC (2011) EDD inhibits ATM-mediated phosphorylation of p53. *J Biol Chem* 286(17):14972–14982.
15. Jung HY, Wang X, Jun S, Park JI (2013) Dyrk2-associated EDD-DDB1-VprBP E3 ligase inhibits telomerase by TERT degradation. *J Biol Chem* 288(10):7252–7262.
16. Maddika S, Chen J (2009) Protein kinase DYRK2 is a scaffold that facilitates assembly of an E3 ligase. *Nat Cell Biol* 11(4):409–419.
17. Hay-Koren A, Caspi M, Zilberberg A, Rosin-Arbesfeld R (2011) The EDD E3 ubiquitin ligase ubiquitinates and up-regulates beta-catenin. *Mol Biol Cell* 22(3):399–411.
18. Honda Y, et al. (2002) Cooperation of HECT-domain ubiquitin ligase hHYD and DNA topoisomerase II-binding protein for DNA damage response. *J Biol Chem* 277(5):3599–3605.
19. Henderson MJ, et al. (2006) EDD mediates DNA damage-induced activation of CHK2. *J Biol Chem* 281(52):39990–40000.
20. Munoz MA, et al. (2007) The E3 ubiquitin ligase EDD regulates S-phase and G(2)/M DNA damage checkpoints. *Cell Cycle* 6(24):3070–3077.
21. Gudjonsson T, et al. (2012) TRIP12 and UBR5 suppress spreading of chromatin ubiquitylation at damaged chromosomes. *Cell* 150(4):697–709.
22. Callaghan MJ, et al. (1998) Identification of a human HECT family protein with homology to the Drosophila tumor suppressor gene hyperplastic discs. *Oncogene* 17(26):3479–3491.
23. Mu JJ, et al. (2007) A proteomic analysis of ataxia telangiectasia-mutated (ATM)/ATM-Rad3-related (ATR) substrates identifies the ubiquitin-proteasome system as a regulator for DNA damage checkpoints. *J Biol Chem* 282(24):17330–17334.
24. Matsuoka S, et al. (2007) ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. *Science* 316(5828):1160–1166.
25. Lim DS, et al. (2000) ATM phosphorylates p95/nbs1 in an S-phase checkpoint pathway. *Nature* 404(6778):613–617.
26. Zhao S, et al. (2000) Functional link between ataxia-telangiectasia and Nijmegen breakage syndrome gene products. *Nature* 405(6785):473–477.
27. Berkovich E, Monnat RJ, Jr, Kastan MB (2007) Roles of ATM and NBS1 in chromatin structure modulation and DNA double-strand break repair. *Nat Cell Biol* 9(6):683–690.
28. Lee JH, Paull TT (2007) Activation and regulation of ATM kinase activity in response to DNA double-strand breaks. *Oncogene* 26(56):7741–7748.
29. Jackson SP, Durocher D (2013) Regulation of DNA damage responses by ubiquitin and SUMO. *Mol Cell* 49(5):795–807.
30. Polo SE, Jackson SP (2011) Dynamics of DNA damage response proteins at DNA breaks: a focus on protein modifications. *Genes Dev* 25(5):409–433.