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Ring Finger Protein RNF169 Antagonizes the Ubiquitin-dependent Signaling Cascade at Sites of DNA Damage*[S]

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Background: Ubiquitylation plays important roles in DNA damage signal transduction. However, mechanistic details that drive these ubiquitin-dependent signals at DNA breaks remain to be determined.
Results: RNF169 localized at DNA double-strand breaks (DSBs), inhibited DNA damage-induced ubiquitin formation, and attenuated 53BP1 accumulation.
Conclusion: RNF169 antagonizes ubiquitin signaling at DNA DSBs.
Significance: RNF169 represents a negative regulator of the ubiquitin-dependent DNA damage signaling cascade.

Ubiquitin signals emanating from DNA double-strand breaks (DSBs) trigger the ordered assembly of DNA damage mediator and repair proteins. This highly orchestrated process is accomplished, in part, through the concerted action of the RNF8 and RNF168 E3 ligases, which have emerged as core signaling intermediates that promote DSB-associated ubiquitylation events. In this study, we report the identification of RNF169 as a negative regulator of the DNA damage signaling cascade. We found that RNF169 interacted with ubiquitin structures and relocalized to DSBs in an RNF8/RNF168-dependent manner. Moreover, ectopic expression of RNF169 attenuated ubiquitin signaling and compromised 53BP1 accumulation at DNA damage sites, suggesting that RNF169 antagonizes RNF168 functions at DSBs. Our study unveils RNF169 as a component in DNA damage signal transduction and adds to the complexity of regulatory ubiquitylation in genome stability maintenance.

Chromatin domains surrounding DNA double-strand breaks (DSBs)[4] are decorated by a plethora of post-translational modifications, including phosphorylation, acetylation, methylation, ubiquitylation, and sumoylation (1, 2). Among these, ubiquitin moieties, including those attached on histone molecules, have emerged as critically important components in orchestrating DNA damage signaling and repair processes (3–5). Indeed, ubiquitylated histones not only are endowed with abilities to alter the physical properties of nucleosomes but are also functionally linked to transcription regulation, DNA damage signal amplification, and DNA repair initiation.

In line with the versatility and reversibility of protein ubiquitylation, recent studies indicated that the ubiquitin landscape at the damaged chromatin is tunable and highly dynamic (6) and consists of an expanding list of DSB-associated ubiquitin conjugates (7–11). Although these different ubiquitin conjugates may govern distinct arms of DNA damage responses, the molecular bases that underlie these regulatory mechanisms remain obscure.

The E3 ligases RNF8 and RNF168 play instrumental roles in propagating DNA damage signals via histone ubiquitylation (12–18). Although RNF8 promotes limited ubiquitylation at DSBs (9, 19), RNF168, upon docking to the RNF8-primed ubiquitin structures, amplifies DNA damage signals by synthesizing ubiquitin chains of Lys^{63} linkages. These allow productive accumulation of tumor suppressors BRCA1 and 53BP1 at DSBs, the failure of which compromises cell cycle checkpoint control and DSB repair. Consistent with pivotal roles in protection of genome integrity, inactivation of RNF168 in mice results in elevated incidence of tumorigenesis (20), highlighting the physiological importance of RNF168-mediated ubiquitylation in genome stability maintenance and tumor suppression.

To further understand how the ubiquitin-dependent signaling pathway is regulated, we undertook a bioinformatics approach and have identified RNF169 as an RNF168-related DNA damage response (DDR) factor. Our results suggest that RNF169 competes for DSB-associated ubiquitin structures and exerts antagonistic effects in DNA damage signal transduction.

EXPERIMENTAL PROCEDURES

Cell Cultures and Transfection—Cells were grown in DMEM supplemented with 10% fetal bovine serum at 37 °C in 5% CO_2. For overexpression studies, cells were transfected with DNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. siRNAs targeting MDC1, RNF8, RNF168,

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[S] This article contains supplemental "Experimental Procedures" and Figs. 1–15.

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[4] The abbreviations used are: DSB, double-strand break; DDR, DNA damage response; MBP, maltose-binding protein; IR, ionizing radiation; IRIF, IR-induced foci; Gy, gray(s).

53BP1, and BRCA1 or control siRNAs were transfected twice at 24-h intervals using Oligofectamine (Invitrogen), and their respective sequences were described previously (12, 21).

Antibodies and Expression Constructs—Antibodies against γH2AX, MDC1, 53BP1, BRCA1, RNF8, and RNF168 were described previously (12, 19). Conjugated ubiquitin was detected by anti-FK2 antibody (Upstate Cell Signaling). Anti-FLAG (M2) and anti-actin antibodies were from Sigma. Anti-KAP1 phospho-Ser^{824} antibodies were from Bethyl Laboratories. Anti-ubiquitin antibodies were from Millipore. Anti-ATM phospho-Ser^{1981} and anti-histone H3 phospho-Ser^{10} antibodies were from Cell Signaling. Anti-DNA-dependent protein kinase phospho-Ser^{2056} antibodies were from Abcam. Rabbit anti-RNF169 polyclonal antibodies were raised against maltose-binding protein (MBP)-RNF169 and purified using a column conjugated with GST-fused RNF169 C-terminal fragment (corresponding to amino acids 334–708). Antibody specificity was confirmed by Western blot experiments using lysates from cells treated with RNF169-targeting siRNAs (supplemental Fig. 1). RNF169 cDNA was subcloned into pDONR201 using Gateway technology (Invitrogen) and was subsequently transferred to Gateway-compatible destination vectors for bacterial or mammalian expression studies. RNF169 mutants were generated using site-directed mutagenesis, and desirable mutations were verified by sequencing.

Interaction Studies—To detect interaction between RNF169 and ubiquitin, MBPs were expressed and purified from BL21 cells using standard procedures and subsequently incubated with monoubiquitin or Lys^{63}-linked ubiquitin chains (Boston Biochem) at 4 °C for 4 h. Protein complexes were washed twice with buffer A (20 mM Tris-HCl (pH 8), 100 mM NaCl, 1 mM EDTA, and 0.5% Nonidet P-40), eluted by boiling in Laemmli buffer, and separated by SDS-PAGE, and immunoblot experiments were performed.

Cell Fractionation Experiments—To assess subcellular localization of RNF168, 293T cells transfected with FLAG-RNF169 or control vector were lysed with buffer B for 10 min on ice. Lysates were cleared by centrifugation, and the supernatant (soluble fraction) was decanted. The remaining pellet was solubilized by incubation with Benzonase nuclease (chromatin fraction; Novagen).

Immunofluorescence Staining—Cells grown as monolayers on coverslips were irradiated at the indicated doses and fixed in 3% paraformaldehyde at 6 h post-treatment unless stated otherwise. Cells were then washed with PBS, permeabilized in 0.5% Triton X-100 solution for 1 min at room temperature, and processed for immunostaining using the indicated antibodies. Nuclei were visualized by staining with DAPI. Images were acquired using an Olympus BX51 fluorescence microscope.

RESULTS

RNF169 Is a DNA Damage-responsive Factor—On the basis of its similar domain organization to the mitotic regulator CHFR, we previously identified RNF8 as a component important for DNA damage signal transduction (12). Using a similar approach, we searched for human proteins that resemble the RIDDLE syndrome protein RNF168 (16, 17), an E3 ligase that amplifies the RNF8-initiated signals at DNA DSBs. This search led to the isolation of RNF169 (Fig. 1*A* and supplemental Fig. 2). Similar to RNF168, RNF169 harbors a RING (really interesting new gene) domain, a signature motif commonly found on E3 ubiquitin ligases. In addition, sequence alignment of RNF168 and RNF169 revealed two putative ubiquitin-interacting domains (MIU1 and MIU2) on the RNF169 polypeptide. Given the similarities between RNF168 and RNF169 and the prominent role of RNF168 in DNA damage signaling, we tested whether RNF169 may also be a component in the mammalian DDR protein network. We first examined whether RNF169 localizes to sites of DNA damage. In unstressed cells, RNF169 proteins were diffusely localized in the nucleus (Fig. 1*B*). Interestingly, ionizing radiation (IR) treatment resulted in robust accumulation of RNF169 at focal structures that overlapped extensively with the DNA damage marker γH2AX (Fig. 1*B*), suggesting that RNF169 may play direct roles in the DDR. Con-

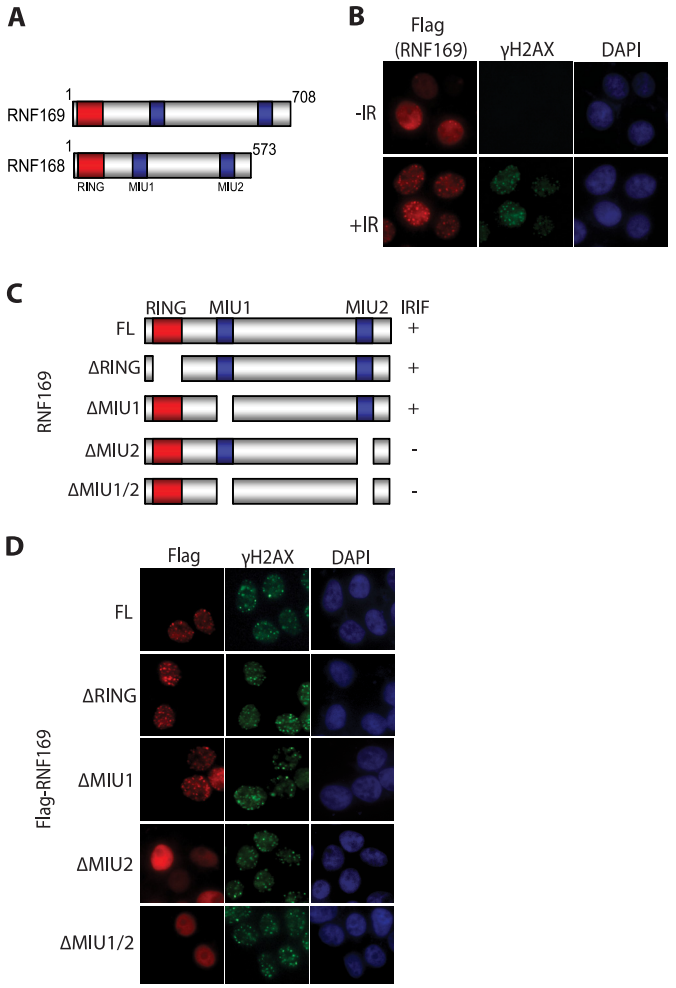

FIGURE 1. **RNF169 is a DNA damage-responsive factor.** *A*, schematic illustration of RNF168 and RNF169 and their domain organization. *B*, RNF169 relocalizes to DNA damage sites following IR treatment. 293T cells transfected with FLAG-tagged RNF169 were either left untreated or irradiated (10 Gy). Subcellular localization of RNF169 was determined by immunostaining experiments using anti-FLAG (M2) and anti-γH2AX antibodies. *C*, schematic illustration of full-length (*FL*) RNF169 and its deletion mutants. A summary of their ability to localize to γH2AX-marked sites is shown on the right. *D*, accumulation of RNF169 at DNA damage sites requires its MIU2 domain. 293T cells expressing the indicated RNF169 constructs were processed to determine their localization following 10-Gy IR treatment.

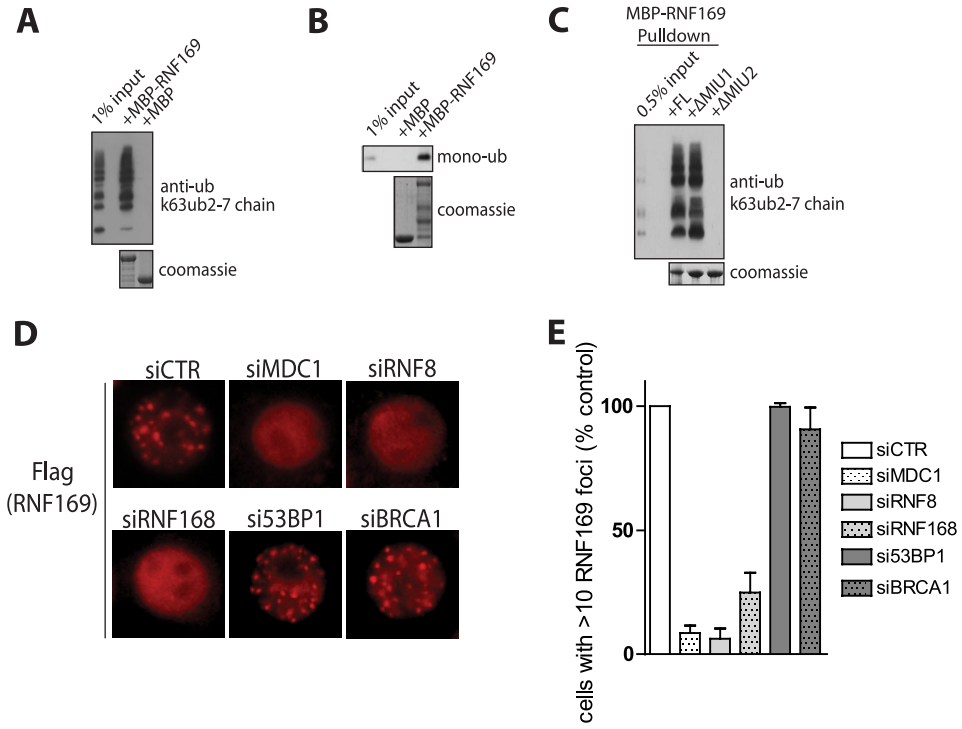

FIGURE 2. **RNF169 accumulation at DNA damage sites requires the RNF8/RNF168-dependent ubiquitin signaling cascade.** *A* and *B*, RNF169 interacts with ubiquitin structures. MBPs were incubated with Lys^{63}-linked ubiquitin chains of 2–7 units (*k63ub2–7*; *A*) or ubiquitin (*B*), and immunoblot experiments were performed using anti-ubiquitin antibodies. *C*, binding of RNF169 to ubiquitin requires its MIU2 domain. MBP-RNF169 or its mutant proteins purified from bacteria were incubated with Lys^{63}-linked ubiquitin chains *in vitro*, and their binding was evaluated by Western blotting. *FL*, full-length RNF169. *D*, cells stably expressing FLAG-RNF169 were transfected with the indicated siRNAs twice at 24-h intervals. Cells were subsequently irradiated (10 Gy), and localization of RNF169 was determined by immunostaining with anti-FLAG (M2) antibodies. *siCTR*, control siRNA. *E*, graphical presentation of the experiments performed in *D* using GraphPad software. Results represent the mean ± S.D. from three experiments, and 100 cells were counted for each experiment.

sistently, endogenous RNF169 proteins also localized to DSBs (supplemental Fig. 3).

Accumulation of RNF169 at DSBs Requires Its MIU2 Domain—To understand how RNF169 relocalizes to DSBs, we generated a panel of deletion mutants and assessed their subcellular localization upon IR treatment (Fig. 1*C*). Accordingly, full-length RNF169 foci were readily detected (Fig. 1*D*). Moreover, IR-induced focal accumulation of RNF169 was not dependent on its RING (ΔRING) or MIU1 (ΔMIU1) domain, as deletion of these domains had no effect on RNF169 localization (Fig. 1*D*). By contrast, deletion of MIU2 (ΔMIU2), as well as the combined deletion of MIU1 and MIU2 (ΔMIU1/2), abrogated RNF169 accumulation at DSBs. Given that MIU2 alone did not accumulate at DSBs (supplemental Fig. 4, *A* and *B*), we concluded that the MIU2 domain is required but not sufficient to target RNF169 to IR-induced foci (IRIF).

RNF169 Interacts with Ubiquitin and Localizes to DSBs in an RNF8/RNF168-dependent Manner—Our observation that IR-induced focal formation of RNF169 required its MIU2 domain prompted us to examine whether RNF169 interacts with ubiquitin. MBP and MBP-fused RNF169 proteins were expressed and purified from bacteria and subsequently incubated with ubiquitin *in vitro*. Pulldown experiments indicated that RNF169 interacted with both Lys^{63}-linked ubiquitin chains (Fig. 2*A*) and monoubiquitin species (Fig. 2*B*). To further test whether the interaction between RNF169 and ubiquitin was mediated via its putative ubiquitin-interacting motifs, we also purified RNF169ΔMIU1 and RNF169ΔMIU2 proteins from bacteria and performed *in vitro* binding experiments. As expected, deletion of MIU2 (but not MIU1) from the RNF169 polypeptide abolished its ability to interact with ubiquitin (Fig. 2*C*). Employing surface plasmon resonance-based technology, we also examined the binding between RNF169 and ubiquitin. Because of the limited solubility of full-length RNF169, we expressed the C-terminal half of RNF169, which contains the ubiquitin-binding MIU2 domain and is localized to DSBs (supplemental Fig. 3), and determined its dissociation constant (K_d = 0.56 μM) with Lys^{63}-linked tetraubiquitin (supplemental Fig. 5).

The RNF8 and RNF168 E3 ligases catalyze local ubiquitylation events at DSBs (5, 22) and are required for the productive accumulation of a number of DNA damage mediator and repair proteins, including 53BP1 (18). Because IR-induced focal formation of RNF169 required its ubiquitin-interacting MIU2 domain, we speculated that RNF169 may localize at DSBs in a manner dependent on the RNF8/RNF168 E3 ligases. Using a panel of previously established siRNAs, we depleted cells of DDR components, including MDC1, RNF8, RNF168, 53BP1, and BRCA1, and determined the subcellular localization of RNF169 upon IR treatment (Fig. 2, *D* and E). Immunostaining analyses revealed that RNF169 IR-induced focal formation required MDC1, an upstream factor essential for the docking of RNF8 and RNF168 at DSBs. Similarly, DSB association of RNF169 was dependent on RNF8 and RNF168, suggesting that ubiquitin structures generated by these E3 ligases may serve as recruiting factors for the accrual of RNF169 at sites of DNA

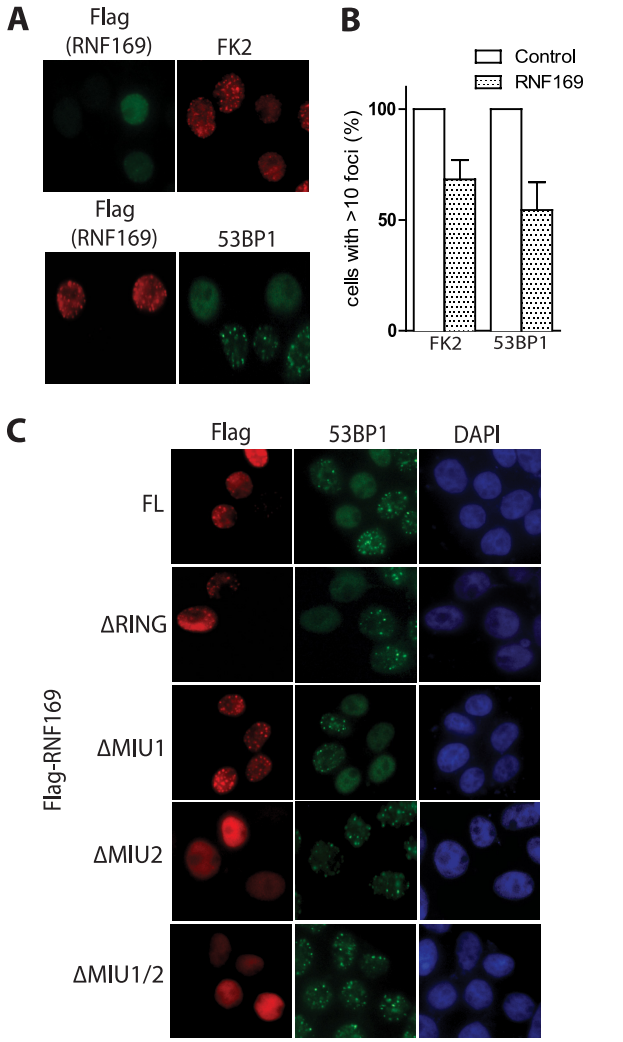

FIGURE 3. **RNF169 attenuates FK2 and 53BP1 accumulation at DNA damage sites.** *A*, 293T cells ectopically expressing FLAG-RNF169 were irradiated (10 Gy). Localizations of 53BP1 and DSB-associated ubiquitin conjugates were determined by immunostaining with the indicated antibodies. *B*, graphical presentation of the experiments performed in *A* using GraphPad software. Results represent the mean ± S.D. from two experiments, and 100 cells were counted for each experiment. *C*, inhibition of 53BP1 DSB accumulation by RNF169 requires its MIU2 domain. 293T cells transfected with the indicated constructs were irradiated (10 Gy), and localization of 53BP1 was assessed by immunostaining experiments as described for *A*. *FL*, full-length RNF169.

damage. Interestingly, knockdown of 53BP1 and BRCA1 did not affect RNF169 localization, indicating that RNF169 may play a role upstream of these tumor suppressor proteins in the DNA damage signaling pathway.

RNF169 Inhibits DSB-associated Ubiquitylation and 53BP1 IRIF—Because RNF169 depletion did not noticeably affect 53BP1 and BRCA1 IRIF (data not shown), the close resemblance of RNF168 and RNF169 prompted us to investigate whether RNF169 may have evolved as a competitor of RNF168 functions. To test this possibility, we ectopically expressed RNF169 and assayed the efficiency of focal accumulation of ubiquitin conjugates (determined by use of anti-FK2 antibodies) and 53BP1 at DSBs, both of which are dependent on RNF168 (16, 17). Remarkably, RNF169 not only attenuated IR-induced FK2 focal formation, it also inhibited accumulation of 53BP1 at DSBs (Fig. 3, *A* and B). These RNF169 inhibitory events were not seen in cells that overexpressed RNF168 (supplemental Fig. 6). Together, these data suggest that RNF169 may limit the RNF8/RNF168-dependent ubiquitin signaling at sites of DNA damage.

Formation of RNF169 IRIF depended on the MIU2 domain (Fig. 1, *C* and D). Consistent with the idea that the function of RNF169 required its DSB localization, introduction of RNF169ΔMIU2 or expression of MIU2 alone had no effect on 53BP1 IRIF (Fig. 3*C* and supplemental Fig. 7). To our surprise, we found that RNF169ΔRING retained its ability to inhibit DSB accumulation of 53BP1. These results indicate that RNF169 may negatively regulate DNA damage signaling by competing for ubiquitin structures at DSBs. Consistent with overexpression studies, RNF169-reconstituted cells exhibited similar domain requirement (*i.e.* MIU2 but not RING) in inhibiting 53BP1 IRIF (supplemental Fig. 8).

RNF169 Antagonizes RNF168 in the DNA Damage Signaling Cascade—The similarities between RNF169 and RNF168 (Fig. 1*A*), together with our observations that RNF169 inhibited FK2 IRIF (Fig. 3, *A* and *B*), led us to test whether RNF169 may limit RNF168 functions in the DDR. Indeed, we found that RNF168 accumulation at DSBs was compromised in cells that ectopically expressed RNF169, whereas RNF8 IRIF were not noticeably affected (Fig. 4, *A* and *B*). To complement these cytological data and to further substantiate the possibility that RNF169 may limit DSB localization of RNF168, we performed cell fractionation experiments and determined the amount of RNF168 proteins on chromatin in the presence or absence of RNF169 overexpression. Although IR treatment induced RNF168 enrichment on chromatin in control cells, this was not observed in cells with ectopic RNF169 expression (Fig. 4*C*).

Inspired by the possibility that RNF169 may antagonize RNF168 function at DSBs, we tested whether down-regulation of RNF169 may restore RNF168 depletion-associated deficits. Given the established role of RNF168 in promoting 53BP1 functions (18), we used the percentage of cells positive for 53BP1 IRIF as a readout. Cells were partially depleted of RNF168 and also treated with either control siRNAs or pooled siRNAs against RNF169. Remarkably, although introduction of RNF169-targeting siRNAs had no effect on 53BP1 focal formation, knockdown of RNF169 in RNF168-depleted cells partially restored 53BP1 focal formation (Fig. 4, *D* and *E*). Used as a control, we found no observable difference in 53BP1 IRIF in RNF8-depleted cells with or without RNF169. Together, our findings support a role of RNF169 in suppressing RNF168-dependent DDR, primarily by competing for ubiquitin structures at DSBs.

RNF169 Depletion Results in Sustained DNA Damage Signaling—On examining the phosphorylation status of a number of DDR factors upon IR treatment, we did not detect requirements of RNF169 in these DNA damage signaling events (supplemental Fig. 9*A*). Similarly, RNF169-depleted cells retained robust G_2/M checkpoint activation (supplemental Fig. 9*B*). These data prompted us to test whether RNF169, as a putative negative regulator of the DNA damage ubiquitin-dependent signaling cascade, may play a more important role in facilitating cell recovery from DDR activation. Following this working model, we hypothesized that these DSB-associated

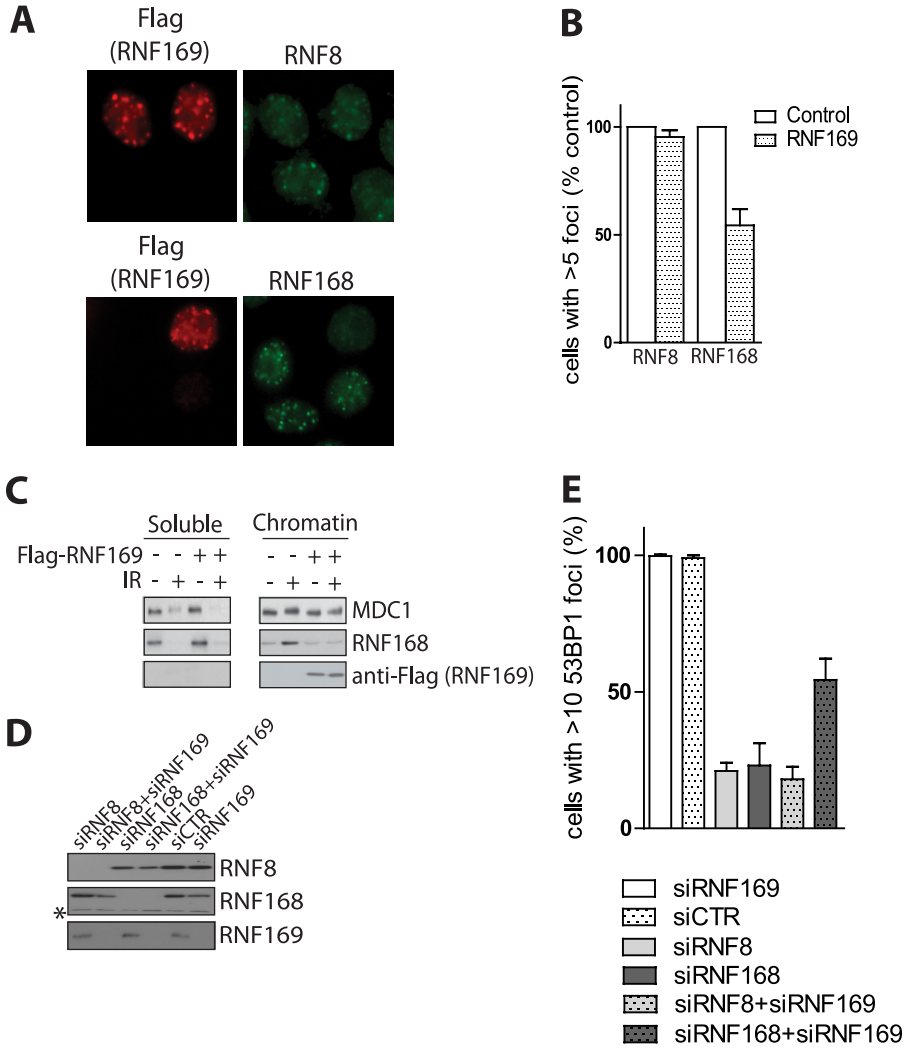

FIGURE 4. **RNF169 perturbs RNF168 localization at DNA damage sites.** *A*, 293T cells ectopically expressing FLAG-RNF169 were irradiated (10 Gy), and subcellular localizations of RNF8 and RNF168 were assessed by immunostaining with the indicated antibodies. *B*, graphical presentation of the experiments performed in *A* using GraphPad software. Results represent the mean ± S.D. from three experiments, and 100 cells were counted for each experiment. *C*, cell fractionation experiments as described under "Experimental Procedures" were performed to determine localization of RNF168 in the presence or absence of ectopic RNF169. *D* and *E*, HeLa cells treated with the indicated siRNAs were either lysed for Western blot analysis (*D*) or processed to determine IR-induced 53BP1 focal formation (*E*). Results represents the mean ± S.D. from two experiments, and 100 cells were counted for each experiment. *siCTR*, control siRNA.

ubiquitin signals may persist in the absence of RNF169. Indeed, not only did we observe sustained 53BP1 and FK2 IRIF in RNF169-depleted cells (Fig. 5*A*), we found that the phosphorylation status of DDR components, including ATM and DNA-dependent protein kinase, persisted in the absence of RNF169 (Fig. 5*B*). Moreover, following the challenge of a recoverable dose of IR (3 grays (Gy)), RNF169-depleted cells displayed prolonged arrest at the G_2/M checkpoint (Fig. 5, *C* and *D*). Conversely, ectopic expression of RNF169 (but not its MIU2 mutant) promoted cell entrance into mitosis following recovery from 3-Gy IR treatment (supplemental Fig. 10).

DISCUSSION

In this study, we have identified RNF169 as a modulator of the RNF8/RNF168-dependent DNA damage signaling pathway. Our results indicated that occupancy of RNF169 at IRIF compromised DSB-associated ubiquitylation and led to defective accumulation of the DNA damage mediator 53BP1 on the damaged chromatin. We propose that RNF169 provides a regulatory mechanism to limit RNF168-mediated signaling, effected primarily via its MIU2-dependent localization and competition for ubiquitin structures at DSBs.

As the primary transducer of DSB-associated ubiquitin signals (9, 19), the RIDDLE syndrome protein RNF168 plays instrumental roles in facilitating 53BP1-mediated DDR functions, the failure of which contributes to immunodeficiency and tumorigenesis (20, 23, 24). Although it remains obscure how the ubiquitin-dependent signaling cascade mediated by the RNF8 and RNF168 E3 ligases promotes 53BP1 accumulation at DSBs, the pivotal importance of protein ubiquitylation in DNA damage signal transduction is underscored by the growing emergence of protein factors that innervate at the level of these core signaling intermediates (11, 25–28). Remarkably, using a bioinformatics approach, we identified RNF169 as a DNA damage-responsive factor that bears high sequence similarities to RNF168 (supplemental Fig. 2). In contrast to the positive role of RNF168 in DNA damage signaling, we found that RNF169 inhibited FK2 and 53BP1 IRIF (Fig. 3). The fact that the RNF169

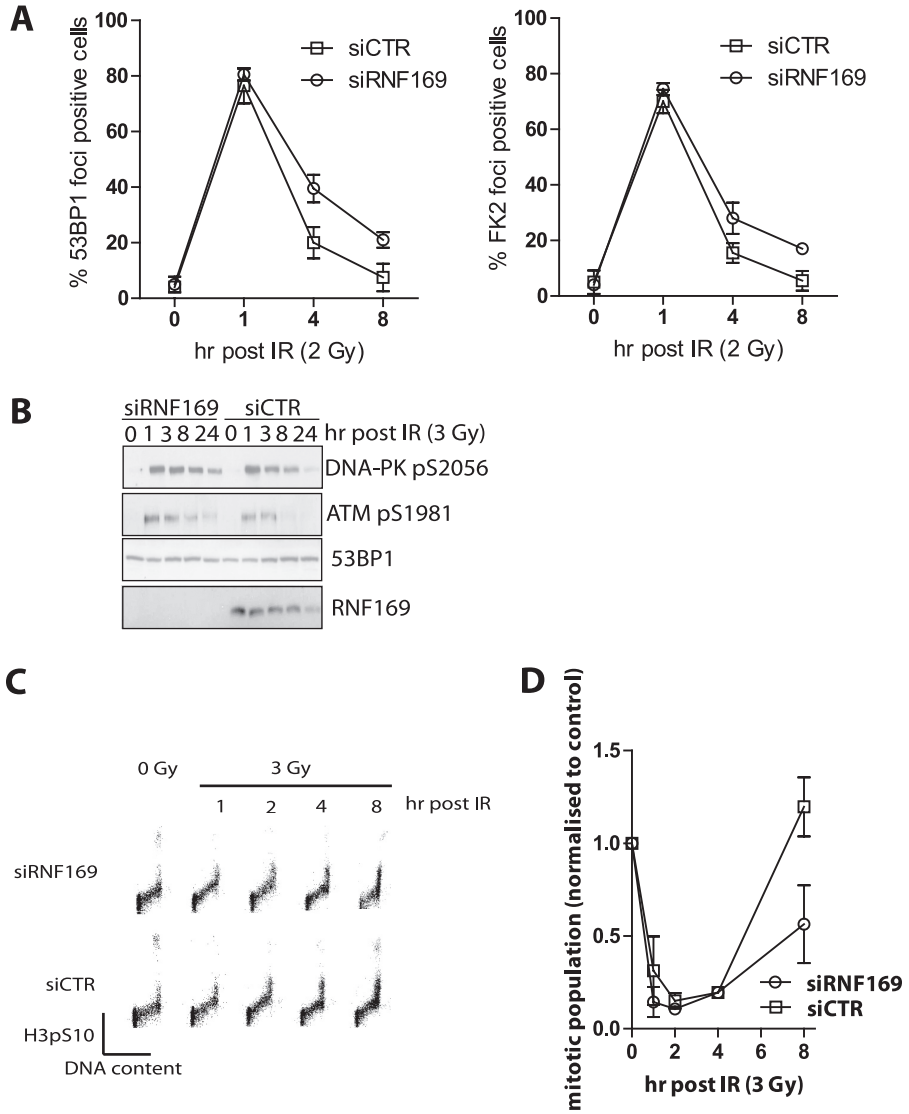

FIGURE 5. **Depletion of RNF169 results in sustained DNA damage signaling.** *A*, HeLa cells treated with the indicated siRNAs were seeded onto coverslips, irradiated (2 Gy), and processed for immunostaining experiments after recovery at the indicated times. Cells positive for 53BP1 and FK2 focal formation (foci > 10 per cell nucleus) were scored, and results from two independent experiments were plotted using GraphPad software. *siCTR*, control siRNA. *B–D*, HeLa cells transfected with RNF169-targeting siRNAs (pooled siRNAs) were irradiated (3 Gy) and processed at the indicated time points for Western blot experiments (*B*) or cell cycle analysis (*C* and *D*). To determine mitotic cell population, cells at the indicated time points were fixed in 70% ethanol, labeled with anti-histone H3 phospho-Ser[10] (*H3pS10*) antibodies, and analyzed using a BD FACSCanto II analyzer. Representative profiles are displayed in *C*, and graphical presentation of the experiments performed in *C* (generated using GraphPad software) shows the average of two independent experiments (*D*). *DNA-PK*, DNA-dependent protein kinase.

RING domain-deleted mutant retained its ability to suppress 53BP1 IRIF (Fig. 3*C*) suggested that RNF169 counteracts DNA damage signal propagation primarily by competing for DSB-associated ubiquitin receptors. Although we cannot exclude a role of the RNF169 RING domain in contributing to its antagonism in DNA damage signaling, it is currently unknown whether RNF169 is a *bona fide* E3 ubiquitin ligase, and the activity of RNF169, if any, does not seem to reply on its RING domain (supplemental Fig. 11) (29). Regardless, given the links between RNF168 inactivation and human diseases, it will be of significant interest to examine whether RNF169 up-regulation may also be associated with DSB repair-associated disorders, including cancers.

Our experiments showing that RNF169 IRIF required the E3 ligases RNF8 and RNF168 suggest that RNF169 may tether to certain ubiquitin conjugates at DSBs (Fig. 2, *E* and *F*). Consistently, not only did IR-induced focal formation of RNF169 require its ubiquitin-interacting MIU2 domain (Fig. 1, *C* and *D*), but its role in inhibiting DSB accumulation of 53BP1 was also perturbed when the MIU2 domain was removed (Fig. 3), highlighting the significance of post-translational protein modifiers in promoting the functional connectivity of the DDR network. Intriguingly, although the MIU2 domain of RNF169 is essential for its targeting to DSBs, RNF169 interacted with RNF168 in a manner that did not require its MIU domains (supplemental Fig. 12). Although we do not understand the significance of this interaction, it is tempting to speculate that RNF169 may also directly regulate RNF168. Future experiments will be needed to test this possibility.

During the preparation of our manuscript, Mailand and coworkers (29) reported similar research findings and concluded that RNF169 represents a negative regulator of the ubiquitin-dependent response to DSBs. Although our data are largely consistent, we found that ectopic expression of RNF169 also perturbed RNF168 IRIF (Fig. 4, *A* and *B*). The reason for this apparent discrepancy is not known. However, given the partial requirement for the MIU2 domain of RNF168 in its targeting to IRIF (16, 17, 30, 31), that RNF168 promotes its own concentration at DSBs (30), and the similarities between RNF168 and RNF169, we favor the idea that RNF169 may occupy certain RNF168-generated ubiquitin adducts that normally allow recruitment of RNF168, 53BP1, and other downstream repair and damage signaling proteins.

Disregulation of DNA damage signaling compromises cell survival and organismal development. Surprisingly, we found that neither overexpression of RNF169 nor its down-regulation elicited detectable changes in cell survival rates using clonogenic survival assays (supplemental Figs. 13 and 14). Given the role of RNF169 in regulating DNA damage signal transduction and checkpoint recovery, it will be of interest to further examine whether aberrant expression of RNF169 may promote genome instability in subtle means that do not affect overall cell survival.

Coordinated deactivation of DNA damage signaling upon completion of DNA repair is key to cell recovery and organismal survival. Whether RNF169 has evolved to set a threshold for the RNF8/RNF168-dependent ubiquitin signaling or whether it serves to actively disarm the DDR will require further experimentations. Although these possibilities are not mutually exclusive, our identification of RNF169 as a negative regulator of the ubiquitin-dependent signaling cascade highlights the complexity of the mammalian DDR (supplemental Fig. 15) and may provide a means to further explore therapeutic approaches to target genome instability-associated syndromes.

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