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RNF169 limits 53BP1 deposition at DSBs to stimulate single-strand annealing repair

Liwei An, Chao Dong, Junshi Li, Jie Chen, Jingsong Yuan, Jun Huang, Kui Ming Chan, Cheng-han Yu, and Michael S. Y. Huen

*School of Biomedical Sciences, Li Ka Shing (LKS) Faculty of Medicine, The University of Hong Kong, Hong Kong S.A.R.; 1Institute of Synthetic Biology, Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences, Shenzhen 518055, P.R. China; 2Department of Radiation Oncology, Center for Radiological Research, Columbia University Medical Center, NY 10032; 3Life Sciences Institute and Innovation Center for Cell Signaling Network, Zhejiang University, Hangzhou, Zhejiang 310058, P.R. China; 4Department of Biomedical Sciences, City University of Hong Kong, Hong Kong S.A.R.; and 5State Key Laboratory of Brain and Cognitive Sciences, The University of Hong Kong, Hong Kong S.A.R.

Unrestrained 53BP1 activity at DNA double-strand breaks (DSBs) hampers DNA end resection and upsets DSB repair pathway choice. RNF169 acts as a molecular rheostat to limit 53BP1 deposition at DSBs, but how this fine balance translates to DSB repair control remains undefined. In striking contrast to 53BP1, ChIP analyses of AssI-induced DSBs unveiled that RNF169 exhibits robust accumulation at DNA end-proximal regions and preferentially targets resected, RPA-bound DSBs. Accordingly, we found that RNF169 promotes CtIP-dependent DSB resection and favors homology-mediated DSB repair, and further showed that RNF169 dose-dependently stimulates single-strand annealing repair, in part, by alleviating the 53BP1-imposed barrier to DSB end resection. Our results highlight the interplay of single-strand annealing repair, in part, by alleviating the 53BP1-imposed barrier to DSB end resection. Our results highlight the interplay of single-strand annealing repair, in part, by alleviating the 53BP1-imposed barrier to DSB end resection. Our results highlight the interplay of single-strand annealing repair, in part, by alleviating the 53BP1-imposed barrier to DSB end resection.

DNA double-strand breaks (DSBs) pose serious threats to genome integrity and cell viability. Unrepaired DSBs not only perturb gene expression programs but can fuel chromosome translocation and chromosome missegregation (1–3), leading to permanent cell arrest, premature cell senescence, and cell death (4). To mend broken DNA in a timely manner, cells deploy multiple DSB repair pathways, namely classical nonhomologous end joining (cNHEJ), alternative nonhomologous end joining (aNHEJ), homology-directed repair (HDR), and single-strand annealing (SSA) (5, 6), to suppress the otherwise deleterious effects of persistent DSBs in cell proliferation and animal development.

cNHEJ represents the predominant DSB repair pathway in mammalian cells, involves no or limited DNA end processing, and does not require sequence homology (7). During cNHEJ, DNA ends are bound by the Ku70/Ku80 complex (Ku) to prevent end resection by nucleases (8). The Ku complex serves as a platform for docking additional NHEJ factors including DNA-PKcs, Artemis, DNA Ligase IV, XRCC4, XLF, and PAXX. By contrast, the aNHEJ, HR, and SSA repair pathways favor resected DSB intermediates that bear 3′ single-strand DNA (ssDNA) overhangs (9). DNA end resection entails the coordinated nucleolytic degradation of 5′ DNA strands and is executed by a cohort of nucleolytic and DNA unwinding activities (10). The mammalian MRE11-RAD50-NBS1 (MRN)/CtIP complex plays an initiating role in DNA end resection and exposes short 3′ ssDNA tails. At this stage, aNHEJ machineries can rejoin broken DSB ends by annealing two ssDNA overhangs that carry microhomologies. Key components of aNHEJ include PARP-1, DNA Ligase III, and DNA polymerase 0. Further DSB processing by EXO1, DNA2, and BLM results in extensively resected ssDNAs that prime HR and SSA repair. HR repair is activated strictly in S and G2 phases of the cell cycle when sister chromatids are available and is considered an error-free repair pathway. In an HR reaction, the RAD51 recombinase nucleates onto ssDNAs at resected DSBs to form nucleoprotein filaments that catalyze homology search and strand invasion events. SSA, however, is adapted to repair DSBs at genomic loci bearing repetitive DNA sequences. The SSA machineries appear to be evolutionarily conserved (11–13) and require extensive DNA end resection to reveal flanking homologous sequence. SSA ensues when the DNA annealing factor RAD52 coats ssDNA overhangs and mediates annealing of DNA molecules that bear homology (14). The nonhomologous 3′ ssDNA tails at the synapsed intermediate are subsequently processed by the ERCC1/XPF endonuclease, and the gaps generated are filled and sealed by uncharacterized DNA polymerases(s) and DNA ligase(s) (5, 15). Notably, SSA is generally considered mutagenic as it is associated with loss of DNA repeats. Notably, aside from the extent of DNA end resection at DSBs, activation and engagement of the mechanistically distinct DSB repair pathways can be influenced by cell cycle phase, DSB chromosomal location, and preexisting epigenetic marks at the DSB landscape (16–18).

53BP1 mediates NHEJ events and is pivotal in programmed DSBs repair, including long-range V(D)J recombination and class-switch recombination (19, 20). 53BP1 is recruited to DSB-flanking chromatin via multivalent interactions involving H2AK15ub (21), H4K20me2 (22), and the nucleosome acidic patch (23), where it has been proposed to protect DSBs from DNA end processing, thereby antagonizing HR and SSA. As such, 53BP1 and its downstream effectors RIFI (24–28), PTIP (29), REV7/MAD2L2 (30, 31), and Shieldin/FAM35A (32, 33) act in concerted efforts to tilt the balance of DSB repair pathway choice in favor of NHEJ. Indeed, unrestrained 53BP1 activities are associated with telomeric fusions and toxic NHEJ repair products (34). Interestingly, not only does 53BP1 inactivation restore HR and contribute to resistance to PARP inhibitors in


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1To whom correspondence should be addressed. Email: huen.michael@hku.hk.

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BRCA cancer cells (35–37), but 53BP1 nullizygosity rescues embryonic lethality of BRCA1-deficient animals (58), highlighting the interplay of 53BP1 and BRCA proteins in DSB repair control. Recent evidence also implies a role of 53BP1 in allowing DSB-flanking chromatin to favor high-fidelity HR by mutagenic SSA (39). Another branch of HR-inhibiting activities is encoded by the RAP80-BRCA1 complex, which accumulates at the DSB-flanking chromatin by recognizing RNF8/RNF168-catalyzed К63-linked ubiquitin structures (40), and is antagonized by ZMYM3 (41). In resemblance to 53BP1, RAP80 docking at DSBs correlates with inhibited DSB resection (42, 43), and RAP80 silencing results in hyperactive HR and SSA (41, 43–46).

The Ring Finger protein RNF169 is emerging as a molecular rheostat that limits 53BP1 and RAP80 deposition at the ubiquitin-modified DSB-flanking chromatin to drive high-fidelity HR repair (47–50). While elegant structural studies have unveiled the mechanistic bases that underlie the functional competition between 53BP1 and RNF169 at DSBs (23, 51, 52), exactly how this fine balance is coupled to the choice of DSB repair pathways remains to be established.

Results

RNF169 Limits 53BP1 and RAP80 Deposition at AsiS1-Induced DSBs. RNF169 suppresses the loading of DNA damage mediator proteins 53BP1 and RAP80 at ionizing radiation-induced foci (IRIF) (47–49). The fact that a subset of RNF169-overexpressing cells still supported formation of 53BP1 IRIF (SI Appendix, Fig. S1A) suggests that RNF169 and 53BP1 may cooccupy DSB-flanking chromatin domains. Analysis of endogenous RNF169 and 53BP1 in IR-treated HeLa cells confirmed that both DNA damage response (DDR) proteins coexist at IRIF (SI Appendix, Fig. S1B). To illuminate the spatial distribution of RNF169 and 53BP1 at IRIF, we visualized IRIF in U2OS cells engineered to stably express Flag epitope-tagged RNF169 using superresolution structured illumination microscopy (SR-SIM). Anti-Flag antibody staining of DIvA-eRNF169 overlapped extensively with RAP80 (Fig. 1C). The differential occupancy of RNF169 and 53BP1 at IRIF was observed in confirmatory studies performed in DIvA-eRNF169 cells pretreated with 0.02 μg/mL doxycycline revealed coexistence of eRNF169 with γH2AX, 53BP1, and RAP80 at AsiS1-induced DSBs (SI Appendix, Fig. S3 C–E). Doxycycline-treated DIvA-eRNF169 cells were subsequently processed to determine the subcellular localization of eRNF169 and its relationship with 53BP1 and RAP80 at AsiS1-induced DNA damage foci using SR-SIM. Consistent with those observed at IRIF (SI Appendix, Fig. S1C), we found that eRNF169 and 53BP1 were often oriented in juxtaposition at individual IRIF. Notably, eRNF169 overlapped extensively with RAP80 (Fig. 1C). The differential occupancy of RNF169 and 53BP1 at AsiS1-induced DSBs was also similarly observed when DIvA-eRNF169 cells were imaged using stochastic optical reconstruction microscopy (STORM; Fig. 1D). Together, these results suggest that RNF169 and 53BP1 may occupy distinct chromatin territories at DSBs.

To test the possibility that RNF169 and 53BP1 may occupy distinct DSB-flanking chromatin domains, we performed chromatin immunoprecipitation (ChIP) experiments to profile the distribution of eRNF169 and a panel of DDR factors at two previously characterized AsiS1-induced DSBs on Chromosome 1 (i.e., Chr1_6 and Chr1_12) (53) (Fig. 1E). Specifically, we determined DDR protein deposition on one side of chromatin (0.1 kb to 2 Mb) flanking each of the two AsiS1 sites. We used previously validated ChIP grade anti-Flag (M2), anti-γH2AX, and anti-53BP1 antibodies (55–57) and our in-house anti-RAP80 antibodies (SI Appendix, Fig. S4 A and B). Accordingly, treatment of DIvA-eRNF169 cells with 4-OHT led to substantial enrichment of γH2AX, 53BP1, and RAP80 at both DSBs compared with control cells (Fig. 1F and SI Appendix, Fig. S5A). Enrichment of γH2AX was detectable to as far as 1 Mb away from each of the AsiS1 sites (Fig. 1F and SI Appendix, Fig. S5A), results which are in line with previous ChIP-sequencing (ChIP-Seq) data that documented megabase spreading of γH2AX along DSB-flanking chromatin (53). Interestingly, whereas the 53BP1 profile along the AsiS1-induced DSB chromatin was similar to that of γH2AX (Fig. 1F and SI Appendix, Fig. S5A), it contrasted with that of RAP80, which preferentially accumulated at DNA ends-proximal regions (Fig. 1F and SI Appendix, Fig. S5A). Notably, the inhibitory effects of RNF169 on 53BP1 and RAP80 accumulation at AsiS1-induced DSBs observed by indirect immunofluorescence staining experiments (SI Appendix, Fig. S2 A and B) can be recapitulated in DIvA-eRNF169 cells pretreated with a high dose of doxycycline (SI Appendix, Fig. S5A). Indeed,
ChIP-qPCR analyses revealed that ectopic expression of RNF169 compromised loading of 53BP1, and to a lesser extent RAP80, onto the damaged chromatin (SI Appendix, Fig. S5A). Intriguingly, eRNF169 displayed robust accumulation at DNA end-proximal regions (SI Appendix, Fig. S5A). However, eRNF169 lacking its MIU2 did not accumulate at AsSI-induced DSBs and did not noticeably affect 53BP1 distribution at the DSB-flanking chromatin (SI Appendix, Fig. S5B), consistent with the requirement of RNF169 MIU2 in its targeting to DSBs (47–49). Importantly, RNF169 inactivation reproducibly led to increase of 53BP1 deposition, but not that of RAP80, at each of the two AsSI-induced DSBs (SI Appendix, Fig. S6 A and B). Together, these results validate the DiVA-eRNF169 cells as a feasible model to study the competitive relationships of RNF169 with 53BP1 at DSBs.
We next pretreated DIvA-eRNF169 cells with a low dose of doxycycline to examine how RNF169 may cooccur with 53BP1 and RAP80 at AsiSI-induced DSBs. At 0.02 μg/mL doxycycline, spatial distributions of γH2AX, 53BP1, and RAP80 at DSB-flanking chromatin were indistinguishable from that of control (Fig. 1F). Notably, we found that eRNF169 was enriched at proximal chromatin regions flanking each of the AsiSI target sites (Fig. 1F), indicating that expression level change of RNF169 did not detectably affect its DSB chromatin distribution. Consistent with our established roles in early events in HR repair, namely DSB end resection and coating of single-strand DNA (ssDNA) overhangs, we found that CtIP, RPA-1, and RAD51 proteins were mostly deposited at DNA end-proximal chromatin regions (SI Appendix, Fig. S7). Together, our ChIP analyses of deposition of DDR factors at AsiSI-induced DSBs uncovered that RNF169 and 53BP1 exhibit dissimilar distribution along the damaged chromatin domains (Fig. 1G) and led us to speculate that the DSB-proximal docking of RNF169 may be important in determining choice of DSB repair pathways.

**Preferential Accumulation of RNF169 at HR-Prone DSBs.** Because RNF169 promotes HR repair (47, 50), we next asked whether RNF169 may preferentially accumulate at HR-prone DSBs by examining RNF169 deposition at RAD51-bound DSBs (DSB IV–VI) and RAD51-unbound DSBs (DSB 1–3) as previously reported (54). We confirmed the preferential deposition of the HR factors RAD51 and RPA-1 at HR-prone DSBs, whereas γH2AX enrichment at both RAD51-bound and RAD51-unbound DSBs were similar (Fig. 2A). Consistent with a role in facilitating HR repair, we found that RNF169 also displayed a preference in binding to RAD51-bound over RAD51-unbound DSBs (Fig. 2A).

**CtIP Facilitates DNA End-Proximal Accumulation of RNF169.** The similar distribution patterns of RNF169 and HR factors CtIP, RPA-1, and RAD51 at AsiSI-induced DSBs prompted us to determine the genetic regulations for RNF169 deposition at DSB-flanking chromatin (Fig. 2B). Although RNF169 distribution differed from that of RNF168 (SI Appendix, Fig. S8), RNF169 enrichment at AsiSI-induced DSBs was hampered following small interference RNA (siRNA)-mediated inactivation of RNF168, consistent with the requirement of the classical RNF8/RNF168-mediated ubiquitination pathway in driving RNF169 IRIF formation (SI Appendix, Fig. S9 A and B). The observation that DSB distribution differed between RNF169 and RNF168 may reflect the maturing of the damaged chromatin, which drives the redistribution of DDR proteins along the DSB-flanking chromatin domains. Given the established roles of the MRN/CtIP complex in DSB end resection, we also

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**Fig. 2.** Characterization of RNF169 end-proximal distribution. (A) RNF169 preferentially loads on RAD51-bound DSBs. DIvA-eRNF169 cells were treated as in Fig. 1F. ChIP-qPCR analyses were performed against γH2AX, RAD51, RPA-1, and Flag (eRNF169) at RAD51-bound and RAD51-unbound DSBs. Box and whisker plots are derived from one representative experiment (n = 3) of three technical replicates. ***p < 0.01, **p < 0.001. (B) Schematic illustrating flow of experiment to define genetic requirement of DSB end-proximal accumulation of eRNF169. (C and D) CtIP promotes RNF169 accumulation at DSB end-proximal regions. DIvA-eRNF169 cells pretreated with indicated siRNAs were incubated with 1.0 μg/mL doxycycline for 24 h. 4-OHT was added to cells for 4 h. Cells were subsequently processed for ChIP experimentations using anti-Flag antibodies (eRNF169). qPCR analysis was performed to determine eRNF169 enrichments at Chr1_6 and Chr1_12. Data represents mean ± SEM (of three technical repeats) derived from one representative experiment (n = 3). *p < 0.05, **p < 0.01. (D) Western blotting experiment was performed to assess RNAi-mediated CtIP knockdown efficiency. (E) CtIP depletion impairs RNF169 accumulation at RAD51-bound DSBs. ChIP-qPCR experimentations and analyses were performed as in C.
tested whether the DNA end resection machineries may contribute to RNF169 accumulation at the DSB-flanking chromatin. To this end, we inactivated MRE11, NBS1, and RAD50 individually in DIVA-eRNF169 cells using the CRISPR/Cas9 method. ChIP of eRNF169 from MRN-inactivated cells did not yield quantitative change to its accumulation at AsiSI-induced DSBs compared with control (SI Appendix, Fig. S9 C and D), despite robust interactions between the MRN complex and RNF169 (SI Appendix, Fig. S9E). We also silenced ChIP using two previously characterized ChIP-specific siRNAs (58, 59) and examined RNF169 loading at the two AsiSI-induced DSBs (Fig. 2 C and D). Interestingly, we reproducibly observed a moderate reduction in RNF169 loading at proximal chromatin regions flanking each of the two DSBs (Fig. 2C). Moreover, accumulation of RNF169 at RAD51-bound DSBs was similarly reduced following ChIP silencing (Fig. 2E). On the contrary, ChIP analysis of CIP, MRE11, and NBS1 at AsiSI-induced DSBs were indistinguishable in control and RNF169 KO cells (SI Appendix, Fig. S10 A–C). Taken together, we propose that ChIP facilitates RNF169 accumulation on chromatin domains proximal to DSBs.

**RNF169 Promotes DSB Resection.** RNF169 enriched at DSB-proximal regions (Fig. 1), accumulated at RAD51-bound DSBs (Fig. 2), and promoted high-fidelity HR repair (47, 50). These observations led us to examine whether RNF169 may be involved in DSB resection. To this end, we quantitatively measured the abundance of ssDNA intermediates at AsiSI-induced DSBs following a previously described method (60). Briefly, genomic DNA harvested from Diva-eRNF169 cells following AsiSI induction was pretreated with restriction enzymes (BstGI or BanI) to digest double-strand DNAs (Fig. 3A). The resulting DNA preparations, including intact resected ssDNA intermediates, were subjected to qPCR quantification. We focused our analysis on two AsiSI target sites on Chromosome 1 (DSB-α) and Chromosome 22 (DSB-β), both of which have been shown to undergo robust DNA end resection (60–62), and assayed for ssDNA intermediates at three loci that span either the BstGI (DSB-α) or the BanI (DSB-β) restriction sites (Fig. 3A). We included an irrelevant site that spans a HindIII restriction site on Chromosome 22 as a negative control (No DSB; Fig. 3A).

Because DSB resection is activated during S/G2 cell cycle phases, to increase DSB resection efficiency and the robustness of ssDNA detection, we briefly arrested Diva-eRNF169 cells at S/G2 cell cycle phases using thymidine before 4-OHT treatment (SI Appendix, Fig. S11A). Accordingly, AsiSI induction resulted in a substantial increase in the abundance of ssDNA intermediates at both “DSB-α” and “DSB-β” sites, but not at the “No DSB” site (Fig. 3 B and C). ssDNA intermediates were more readily detected at DSB proximal regions, consistent with the nature of DSB resection regulation (Fig. 3 B and C). Inactivation of ChIP by siRNAs suppressed ssDNA generation, consistent with its roles in initiation of DSB resection (63) (Fig. 3 B–D). We further tested the effects of BRCA1 and EXO1 depletion on ssDNA generation since both DDR factors are associated with the DSB resection process (64, 65). Results showed that the ssDNA generation was modestly but significantly decreased in BRCA1-depleted or EXO1-depleted cells (Fig. 3 B–D). Notably, RNF169 depletion by two independent previously characterized siRNAs (48) similarly led to reduction in the abundance of ssDNA intermediates at the AsiSI-induced DSB sites, indicating that RNF169 may facilitate DSB end resection (Fig. 3 B–D). To further corroborate this idea, we generated RNF169 knockout (KO) AsSI−/−B1U20 cells and found that level of ssDNA in intermediates was also modestly reduced (SI Appendix, Fig. S11 B–D). By contrast, deficiency of both 53BP1 and RAP80 reproducibly led to increased abundance of ssDNA intermediates (SI Appendix, Fig. S11 B–D), results that are in line with their established roles in limiting DSB resection (24, 43, 45, 60). We concluded that RNF169 promotes DSB end resection.

**RNF169 Promotes Homology-Mediated DSB Repair.** We next assessed the roles of RNF169 in DSB repair using established cell reporters that measure DSB repair events mediated by HR, SSA, and aNHEJ and total NHEJ, respectively (66). We considered the putative role of RNF169 in facilitating DSB end resection (Fig. 3 B and C and SI Appendix, Fig. S11 B and C), we speculated that RNF169 may be specifically required for resection-dependent DSB repair, namely, HR, SSA, and aNHEJ. Accordingly, the DSB repair reporter cells harbor a disrupted GFP gene, and expression of intact GFP requires successful repair of an F-Scel–induced DSB at the gene locus, and can be quantified by flow cytometric analysis (Fig. 3 E–H, Top) (66). We silenced RNF169 using two individual siRNAs (48), and found that RNF169 inactivation compromised high-fidelity HR repair, results that are entirely consistent with previously described (47, 50). The core HR factor PALB2 served as a positive control (Fig. 3E). Importantly, SSA and aNHEJ repair efficiencies were also significantly reduced following RNF169 silencing, as were that in RAD52-depleted and Polδ-depleted cells, which were used as positive controls to assess SSA and aNHEJ, respectively (Fig. 3 F and G). By contrast, we did not observe robust change in NHEJ repair efficiency in RNF169 knockdown cells (Fig. 3H). Together, these data suggested that RNF169 promotes (micro)homology-directed DSB repair, consistent with its role in facilitating DSB resection.

**RNF169 Dose-Dependently Regulates SSA Repair.** Inspired by the role of eRNF169 in limiting DSB deposition of 53BP1 and RAP80, we tested whether forced expression of RNF169 may overcome the 53BP1-imposed and RAP80-imposed barrier to resection-dependent DSB repair processes. To this end, we overexpressed RNF169 in the DSB repair reporter cells, and surprisingly, found that RNF169 specifically stimulated SSA repair (Fig. 4 A and B and SI Appendix, Fig. S12 A–C). RNF169-driven SSA repair also required its MIU2 domain (Fig. 4 A and B), suggesting that RNF169 promotes SSA by antagonizing 53BP1, and that 53BP1 supported hyperactive SSA (Fig. 4 C and D) (68), we tested whether 53BP1 inactivation may reverse the stimulating effect of RNF169 on SSA. Indeed, we found that the RNF169-stimulated SSA was largely attenuated in 53BP1 KO cells (Fig. 4 E and F), indicating that RNF169 promotes SSA, at least in part, by counteracting 53BP1.

We further examined whether 53BP1 deficiency may restore SSA repair to normal levels in a RNF169-deficient background. Consistent with the idea that the RNF169-53BP1 balance modulates SSA repair, we found that coexpression of RNF169 and 53BP1 restored SSA compared with otherwise wild-type conditions (Fig. 4 C and F). It is noteworthy to mention that 53BP1 deficiency did not alleviate the HR defects seen in RNF169 knockdown cells (SI Appendix, Fig. S13 A and B), highlighting a more specific role of 53BP1 in counteracting RNF169-dependent SSA repair.

**RNF169 Stimulates SSA Repair in HR-Deficient Cells.** Given that 53BP1 loss alleviates defects in DSB resection in BRCA cells (35–37), our observation that RNF169 may regulate SSA repair by counteracting 53BP1 activity led us to examine the regulation of RNF169-driven SSA in BRCA1-deficient, BRCA2-deficient, and PALB2-deficient cells. We therefore tested for a more specific role of RNF169 in modulating SSA repair in BRCA1-deficient backgrounds. Accordingly, BRCA1 silencing impaired SSA (Fig. 5), consistent with its roles in multiple steps of DSB resection and DSB repair pathways (69). Inactivation of the core HR factor BRCA2 and PALB2, however, resulted in hyperactive SSA (Fig. 5), an observation that is in line with the competition between SSA and HR (70, 71). Interestingly, consistent with its dose-dependent stimulating effect on SSA repair, cells overexpressing RNF169 supported substantially elevated SSA in BRCA1-silenced, BRCA2-silenced, and PALB2-silenced cells (Fig. 5 A and B), whereas inactivation of RNF169 in BRCA2-silenced and PALB2-silenced cells, but not BRCA1-deficient cells, led to marked reduction of the otherwise hyperactivated SSA (Fig. 5 C and D). Together with the observation where forced expression of RNF169 did not affect HR repair in BRCA1, PALB2, or BRCA2 knockdown cells.
Fig. 3. RNF169 promotes DSB resection and homology-directed repairs. (A) Schematic for quantitative DNA resection assay based on the DIvA system. (B and C) Quantitative measurement of ssDNA generation by S' end resection at two AsSI-induced DSBs. DIvA cells pretreated with indicated siRNAs were incubated with 4-OHT for 4 h. Genomic DNA was extracted and digested with either BsrGI (B) or BanI (C). Percentage of ssDNA intermediates at indicated sites was measured by qPCR using primers indicated in A after restriction enzyme digestion. Data represents mean ± SEM (of two technical repeats) from three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001; ns, not significant. (D) Western blotting experiment was performed to assess RNAi-mediated knockdown efficiency in cells used in B and C. (E–H) RNF169 deficiency impairs resection-dependent DSB repair. Schematic representation of the DR-GFP, SA-GFP, EJ2-GFP, and EJ5-GFP reporters to analyze the repair of I-SceI-induced DSBs by HR, SSA, aNHEJ, and total NHEJ events (Top). U2OS cells stably expressing DR-GFP (F), SA-GFP (E), EJ2-GFP (G) and EJ5-GFP (H) were transfected with indicated siRNAs and were electroporated with plasmid encoding the I-SceI endonuclease. (Middle) Flow cytometric analysis of GFP-positive cell population was performed 48 h after electroporation. Data represents mean ± SEM from three independent experiments, **P < 0.01, ***P < 0.001; ns, not significant. (Bottom) Knockdown efficiencies were determined by Western blotting.

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Role of RAP80 in RNF169-Dependent DSB Repair. Our ChIP profiling and superresolution imaging experiments revealed overlapping distribution of RNF169 and RAP80 at DSB-flanking chromatin domains (Fig. 1 C and F). In addition to its documented roles in restricting DSB resection (41, 43), RAP80 knockdown cells also displayed hyperactive HR and SSA repair (41, 43–45). Because eRNF169 also limited RAP80 deposition at DSBs (SI Appendix, Figs. S2 B and S4A), we studied the antagonistic relationships between RNF169 and RAP80 in DSB repair cell. We inactivated RAP80 using two independent RAP80-targeting gRNAs (72) and found that RAP80 deficiency coincided with hyperactive HR, SSA, and aNHEJ repair (SI Appendix, Fig. S14 A–C). Importantly, inactivation of RNF169 partially but significantly dampened HR, SSA, and aNHEJ repair in RAP80 null cells (SI Appendix, Fig. S14 A–C). Because both 53BP1 and RAP80 limits DSB end resection, and that forced expression of RNF169 specifically promoted SSA repair (Fig. 4A), we examined whether the RNF169-driven SSA may be attenuated in cells deficient in both 53BP1 and RAP80. Consistently, we found that cells inactivated for both RAP80 and 53BP1 effectively suppressed the stimulating effect of RNF169 in SSA (SI Appendix, Fig. S14 D and E), revealing a complex interplay between RNF169 and 53BP1/RAP80 in DSB repair control.

Discussion

The RING finger protein RNF169 counteracts the loading of DNA damage mediator proteins 53BP1 and RAP80 onto DSBs and has emerged as a negative regulator in DSB signal transduction (47–49). However, exactly how RNF169 fine-tunes 53BP1 and RAP80 activities to execute DSB repair has remained unknown. On the basis of the RNF169-encoded antagonism of 53BP1 and RAP80, our observations that dosage imbalance of RNF169 dysregulates DSB resection and choice of DSB repair pathways suggest that RNF169 may skew DSB repair pathways, at least in part, by restraining 53BP1-dependent and RAP80-dependent signal amplification. Indeed, the notion that DSB signal output, including the extent of DSB ubiquitylation, plays a determining role in choice of DSB repair in not unprecedented (73, 74). Previous work has identified OTUB2 as a negative regulator of the core ubiquitin ligase RNF8, where it suppresses RNF8 activity to promote high-fidelity HR repair (75). The importance of maintaining optimal RNF8 output was also unveiled recently with the identification of the E3/E4 ligase UBE4A, which enforces DSB signal output to promote optimal DSB resection and HR repair (76). Although it remains to be seen if RNF169

(SI Appendix, Fig. S13 C and D), our findings suggest that RNF169 specifically fine-tunes SSA repair in BRCA cells.

Fig. 4. RNF169 stimulates SSA repair by counteracting 53BP1. (A) Ectopic expression of RNF169 but not RNF169MIU2 stimulates SSA repair. SA-U2OS-eRNF169 or SA-U2OS-eRNF169MIU2 cells were electroporated with plasmid encoding the I-SceI endonuclease. Cells were cultured with or without 2 μg/mL doxycycline for 48 h before cells were harvested for flow cytometric analysis. Mock electroporation (no I-SceI) was used as negative control. Data represents mean ± SEM from three independent experiments, ***P < 0.001, ns, not significant. (B) Western blotting analysis showing expression of RNF169 (wild-type and ΔMIU2 mutant). (C) 53BP1 inactivation promotes SSA repair. SA-U2OS (vector control and 53BP1 KO) cells were electroporated with I-SceI expression construct and percentage of cells positive for GFP was analyzed 48 h after electroporation. Data represents mean ± SEM from three independent experiments, ***P < 0.001, ns, not significant. (D) Western blotting analysis showing expression of 53BP1 deficiency restores SSA repair in RNF169-inactivated cells. Parental SA-U2OS cells (Vector) or its 53BP1 KO derivative were treated and processed as described in A. Data represents mean ± SEM from three independent experiments, ***P < 0.001, ns, not significant. (E) Western blotting analysis to determine RNF169 expression in cells used in E. (G) 53BP1 deficiency restores SSA repair in RNF169-inactivated cells. Parental SA-U2OS cells (Vector) or its 53BP1 KO derivative were transfected with indicated siRNAs. Cells were treated and processed as described in C. Data represents mean ± SEM from three independent experiments, ***P < 0.001. (H) Western blotting analysis was performed to assess RNAi-mediated knockdown efficiency in cells used in G.

Fig. 5. RNF169 stimulates SSA repair in HR-deficient cells. (A and B) Ectopic expression of RNF169 stimulates SSA repair in BRCA1-deficient, PALB2-deficient, and BRCA2-deficient cells. SA-U2OS-eRNF169 cells pretreated with indicated siRNAs were transfected and processed as described in Fig. 4A. Data represents mean ± SEM from four independent experiments, ***P < 0.001, ***P < 0.001. (B) Western blotting experiment was performed to assess RNAi-mediated knockdown efficiencies. (C and D) RNF169 is required for hyperactive SSA in PALB2-deficient and BRCA2-deficient cells. SA-U2OS cells were transfected with indicated siRNAs and processed as described in Fig. 4C. Data represents mean ± SEM from four independent experiments, **P < 0.05, ***P < 0.001; ns, not significant. (D) Western blotting experiment was performed to assess RNAi-mediated knockdown efficiencies.
may have additional roles in DSB response control, our data adds an additional regulatory layer of DSB signal output and firmly establish the interplay of RNF169 and 53BP1/RAP80 as active regulators of DSB repair pathway choice. Current evidence suggests that both RNF169 and 53BP1 recognize and compete for H2AK15ub-containing nucleosomes at DSBs, with RNF169 bearing higher affinity for the ubiquitin conjugate in vitro (21, 51, 52). However, exactly how the two competing activities engage in a dynamic interplay at the damaged chromatin is not clear. By recapitulating the dynamic antagonisms of RNF169 and 53BP1 at IRES in the DIVA platform (SI Appendix, Figs. S2 A and B and S5A), we employed super-resolution imaging to capture RNF169 and 53BP1 at AsiSI-induced DSBs and have revealed that RNF169 is oriented juxtaposed to 53BP1 (Fig. 1 B and D), an observation supported by our ChIP experiments (Fig. 1F). Interestingly, ChIP profiling of RNF169 and 53BP1 at AsiSI-induced DSBs not only indicates that RNF169 and 53BP1 exhibit dissimilar distributions at the DSB-flanking chromatin, but also revealed that RNF169 preferentially accumulates at chromatin territories proximal to the DSBs, raising the possibility that RNF169 may contribute to the early processing of DSBs. In support of this idea, we found that RNF169 inactivation attenuated DSB end resection (Fig. 3 B–D and SI Appendix, Fig. S11 B–D) and led to impaired DSB repair, including SSA (Fig. 4 E–G). The fact that forced expression of RNF169-stimulated SSA repair (Figs. 4 A and E and S/A) but not HR or aNHEJ (SI Appendix, Figs. S9 A and B and S13C) suggests that DSB processing, especially involving long-range DNA resection, may be more dependent on the homeostatic balance of RNF169 and 53BP1 at DSBs. This idea is supported by the observation that RNF169 silencing, much like inactivation of EXO1, did not noticeably affect CPT-induced RPA-1 foci detected by indirect immunofluorescence studies (SI Appendix, Fig. S15 A–C). Moreover, dosage imbalance of RNF169 also did not dysregulate local curvature or distribution of BRCA1 and RAD51 at AsiSI-induced DSBs (SI Appendix, Fig. S16 A–C). Considering that 53BP1 prevents hyperresection to foster high-fidelity DSB repair (39), and that 53BP1 deficiency attenuated RNF169-driven SSA (Fig. 4E), our findings highlight the importance of a fine balance of RNF169 and 53BP1 in proper choice of DSB repair pathways, and that RNF169 amplification may contribute to genome instability in human cancers (SI Appendix, Fig. S17).

Unlike the established competition between RNF169 and 53BP1 for H2AK15ub (21, 51, 52), exactly how RNF169 displaces RAP80 from DSBs is less clear. Since RNF169 promotes its own DSB recruitment by interacting with H2AK15ub (49, 51, 52), one can envisage that RNF169 may compete with RNF168 for H2AK15ub-containing nucleosomes, thereby suppressing the RNF168-dependent RAP80 recruitment onto the damaged chromatin. Alternatively, that RAP80 is targeted to DSBs via its ability to interact with both SUMO-linked and K63-linked ubiquitin polymers (77–81) raises the possibility that RNF169 may bind to additional but heretofore unidentified targets at DNA end-proximal regions surrounding DSBs, given our observations that RNF169 and RAP80 exhibit similar distribution patterns along chromatin domains that flank DSBs (Fig. 1F).

Intriguingly, although RNF169 docks at IRESs and AsiSI-induced DSBs in manners that strictly require its ubiquitin-binding MIU2 domain and the primary DSB ubiquitin ligases RNF8 and RNF168 (47–49) (SI Appendix, Figs. S2D, SSB, and S9 A and B), we found that ChIP inactivation specifically attenuated RNF169 acclimat at chromatin domains proximal to DSBs (Fig. 2 C–E), considering that RNF169 interacts with the MRN complex (SI Appendix, Fig. S9E), and that RNF169 promotes DSB end resection (via Fig. 3 B–D and SI Appendix, Fig. S11 B–D). It is tempting to speculate that the MRN/ChIP-RNF169 complex may contribute to the optimal processing of DNA ends. Although further experiments will be required to clearly define how the MRN/ChIP-RNF169 axis contributes to optimal DNA resection, our work suggests that RNF169 shunts DSBs to resection-dependent repair, and that aberrant RNF169 expression may upset proper DSB repair pathway choice and contribute to chromosomal instability (SI Appendix, Fig. S18 A–C).

While the tumor suppressor BRCA1 may participate in multiple DSB repair pathways (69), consistent with previous reports (70, 82–84), we found that inactivation of the core HR factor PALB2 and BRCA2 led to substantially elevated SSA (Fig. 5), highlighting the complex and competitive nature of HR and SSA. Our observation is in line with the competing nature of the resection-dependent DSB repair pathways, where inactivation of HR machineries, including BRCA2 and RAD51, has been reported to fuel aNHEJ and SSA (82, 83, 85). Importantly, RNF169 promoted SSA in both PALB2 and BRCA2 proficient and deficient backgrounds (Fig. 5), highlighting an unprecedented role of RNF169 as an SSA factor. Because SSA plays an important role in sending DNA DSBs at repetitive DNA loci, noting the increased repetitive nature of higher eukaryotic genomes, it would be of significant interest to explore how RNF169 may have evolved as an RNF169 paralogue, how RNF169 supports DSB response control, and implicate RNF169 as a key component in the mammalian DDR network that safeguards genome stability in higher eukaryotes (SI Appendix, Fig. S20).

Materials and Methods

Cell Cultures. AsiSI-ER-U2OS cells were cultured in DMEM supplemented with 10% FBS and 1 μg/mL puromycin at 37 °C in 5% CO2. For AsiSI-dependent DSB induction, cells were treated with 600 nM 4-OHT (H7904; Sigma) for 4 h. The four DSB repair reporter cell lines (EJ2-U2OS, DR-U2OS, SA-U2OS, and EJS-U2OS) were cultured in DMEM supplemented with 10% FBS and were previously described (66). For generation of cell lines with doxycycline-inducible expression of RNF169 (WT and ∆MIU2), the RNF169 cDNA was cloned in frame 3′ of the SFB (S protein, Flag, and Streptavidin-binding peptide) sequence. Cells (DIVA and the four reporter cell lines) were infected with lentiviral particles carrying RNF169 (WT and ∆MIU2)-SFB expression constructs and were subsequently selected by 2 μg/mL G418 for 1 wk. Expression of RNF169-SFB was induced by supplementing cell culture media with doxycycline (D9891; Sigma).

Generation of KO Cells by Use of the CRISPR/Cas9 Method. All KO cell lines (DIVA, DR-U2OS, and SA-U2OS) used in this study were generated using the CRISPR-Cas9 gene targeting approach. All of the guide RNAs (gRNAs) used in this study were cloned into the p lentirCRISPR v2 vectors and their sequences are as follows: MRE11 gRNA: 5′-GCAUCUCCGAAGAACUAAA-3′; CtIP gRNA2#: 5′-CATCCTCTAGAACC-3′; RNF169 siRNA-1#: 5′-CAA-3′; RAD52 siRNA: 5′-ACTCGGCTGTGAGTGAGTTA-3′; NBS1 gRNA: 5′-GTAACCGCGGCTCTGGAC-3′; Polθ gRNA#: 5′-TAATAAGGGTTGAGGAAATATC-3′; DNA Ligase IV siRNA: 5′-GGAGUGACUCAAGAAUUAA-3′; CtIP siRNA-2#: 5′-CTCGGCGCTCTCGTCAAGC-3′; POLQ siRNA-2#: 5′-GAAAGAATACCTGAGTTTGC-3′. For generation of KOs, cells were infected with lentiviral particles harboring each gRNA twice at 24-h intervals. KO cells were allowed to grow for 1 wk before validation by Western blotting.

Lentivirus Packaging and Infection. Lentiviral particles were produced by transiently cotransfecting the lentiviral-based expression constructs together with packaging plasmids psPAX2 and pMD2.G at a ratio of 3:1 into HEK293T cells. Forty-eight hours after transfection, the supernatants were filtered (0.45 μm) and were applied to recipient cell lines in the presence of 8 μg/mL polybrene (Sigma).

RNA Interference. For RNAi-mediated depletion experiments, cells were transfected with two forms of either nonrepeating target or targeting siRNAs (Dharmacon) using Oligofectamine (Invitrogen). siRNAs against RNF168 were previously described (86, 87). Other target genes and their siRNA sequences are as follows: Control siRNA: 5′-UGAGGCGAUAACACAAU-CAA-3′, RNF169 siRNA-1#: 5′-GAGGCGACUUAAUUAAUUC-3′, RNF169 siRNA-2#: 5′-GAGGCGACGUAAUAAUAAU-3′ (SI Appendix, Fig. S6); CtIP siRNA-1#: 5′-UGGAGAAGAACAAUCA-UCUAAU-3′; CtIP siRNA-2#: 5′-AAGACGAAACAGGAAAGAU-3′; PALB2 siRNA: 5′-GGAUAAACUAAUCCUGGAA-3′; RAD2 siRNA: 5′-GAGGAGCUACCCAGAAUUAAU-3′; DNA Ligase IV siRNA: 5′-AAGCCGACAAACAGGUGGA-3′; Polθ siRNA: 5′-AGGCUCAUCGAAAGAAGGGA-3′; BRCA1 siRNA: 5′-GAACATGTCCTCAACCAAAAG-3′.
Antibodies used for immunofluorescence staining, Western blotting, and ChiP are listed in SI Appendix, Table S1.

Immunofluorescence Staining. Cells grown on coverslips were fixed with 3% formaldehyde for 15 min at room temperature followed by permeabilization in 0.5% Triton solution for 30 s. A preextraction step was used when necessary and involved cell premeabilization in Triton solution for 30 s before fixation. After blocking with 3% milk at room temperature for 20 min, cells were stained by sequential incubation of primary antibodies and secondary fluorophore-conjugated antibodies (SI Appendix, Table S1). DAPI was used to stain nuclear DNA. Images were captured using a 60× oil immersion lens on an Olympus BX51 fluorescence microscope and were further processed by ImageJ software.

Superresolution Fluorescence Microscopy. SR-SIM imaging was performed on a Zeiss Elyra S1 microscope with 100× oil immersion lens (N.A. = 1.46). Z-stack images with 0.1 μm per step were taken between 1 and 2 μm over the glass substrate. SR-SIM images were reconstructed by Zeiss Efficient Navigation (ZEN) software, and maximum projection of the entire 1-μm volume was processed by ImageJ software. Dual-color STORM images were acquired by a SRIS (Super-Resolution Microscope (Nano BioImaging) and processed with QuickPALM in ImageJ, as previously described (88).

ChiP. ChiP was performed based on a protocol previously described with minor modifications (89). Briefly, cells were cross-linked with formaldehyde (1.42%) for 15 min at room temperature. Cross-linking was quenched by the addition of glycine (125 mM) for 5 min. Cells were washed twice with cold PBS and collected by scraping. Pelleted cells were resuspended in 300 μL of lysis buffer (50 mM Hepes/KOH, pH 7.5; 50 mM Hepes/KOH, pH 7.5; 140 mM NaCl; 1 mM EDTA; 1% Triton X-100; 0.1% Na-deoxycholate and protease inhibitors). To shear the chromatin, cells were sonicated with Bioruptor for 18 cycles (high power, 30 s on and 30 s off). After sonication, samples were diluted twofold with a fresh lysis buffer and subsequently centrifuged to clear the supernatant. Fifty microliters of supernatant were directly processed to extract total DNA as whole cell input. The remaining supernatants were transferred to new Eppendorf tubes and were incubated with indicated antibodies (SI Appendix, Table S1) together with prewashed Protein A beads (16 μL of 10% Chelex (1421253; Bio-Rad). The samples were boiled for 10 min and centrifuged again to spin down the beads. Combine the supernatants together as templates for follow-up qPCR analysis.

Real-Time qPCR Analysis. PCR analysis was performed on a MyiQ2 Real Time PCR Detection System (Bio-Rad) using the iTaq SYBR Green Supermix (172–5124; Bio-Rad), according to the manufacturer’s instructions. All samples were analyzed in duplicates. The IP efficiency was calculated as percent of input for DNA immunoprecipitated. Primer sequences used for profiling protein distribution at Chr1, 6 and Chr1_12 were listed in SI Appendix, Table S2. Primers used for qPCR analysis at RAD51-bound and RAD51-unbound DSBS were described (54).

In Vivo DNA End Resection Assay. Briefly, DIVa cells were treated with or without 600 mM 4-OHT for 4 h to induce AsSi-dependent DSBS. Thereafter, genomic DNA was purified using the standard phenol-chloroform extraction method. For each sample, around 300 ng of extracted DNA was subjected to an Rnase H treatment for 15 min before mock digestion or digestion with 20 units of BsrGI (DSB−; BsrGI (DSB−; HindIII) (No DSB) at 37 °C overnight. Samples were heat-inactivated at 65 °C for 10 min and were analyzed by qPCR. To quantify the extent of resection, around 20 ng (2 μL) of mock digested or indicated restriction enzyme digested samples were amplified by qPCR using primers listed in SI Appendix, Table S3. The percentage of ssDNA (ssDNA %) was calculated based on the following equation: ssDNA % = (1/(2−Ct+1.5) × 0.5) × 100. Ct was calculated by subtracting the Ct value of the mock-digested sample from the Ct value of indicated restriction enzyme digestion sample. At least three biological replicates were performed.

Cell Cycle Analysis. To collect cells in different cell cycle phases, cells were treated with mimosine (M0253; Sigma) for 24 h to arrest cells in G1 phase. S and G2 phase cells were collected 7 h and 15 h upon mimosine release, respectively. To directly collect G2 cell populations, cells were treated with 2 mM thymidine for 18 h and further released for 4 h before harvest. Cells were trypsinized and fixed with cell and fixed with cold-70% ethanol. After overnight incubation at −20 °C, fixed cells were washed once with PBS and were treated with 200 μL of sodium citrate solution containing Rnase A for 30 min at room temperature followed by addition of another 200 μL of sodium citrate solution containing 50 μg/mL propidium iodide. Cell-cycle distribution was determined using a BD FACS Cantor Analyzer.

DSB Repair Analysis in the NR, cNHEJ, SSA, and aNHEJ Reporter Cell Lines. U2OS cells stably expressing DR-GFP (DR-U2OS), EJSS-GFP (EJS-U2OS), SSA-GFP (SSA-U2OS), and EJZ-GFP (EJZ-U2OS) (67) were transfected with indicated siRNAs and electrooporated with the i-Scel expression construct (pcBASCe) at 200 V, 975 microfarads using Gene Pulser Xcell (Bio-Rad). Cells were further recovered for 48 h after electrooporation and were subjected to flow cytometric analysis using a BD FACS Cantor Analyzer.

Statistical Analysis. Unless otherwise stated, data represent mean ± SEM of at least three independent experiments. Student’s t test (two-tailed) was used to evaluate statistical significance, and a P < 0.05 value was considered as significant.

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