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The RPA–RNF20–SNF2H cascade promotes proper chromosome segregation and homologous recombination repair

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The human tumor suppressor Ring finger protein 20 (RNF20)-mediated histone H2B monoubiquitination (H2Bub) is essential for proper chromosome segregation and DNA repair. However, what is the precise function and mechanism of RNF20–H2Bub in chromosome segregation and how this pathway is activated to preserve genome stability remain unknown. Here, we show that the single-strand DNA-binding factor Replication protein A (RPA) interacts with RNF20 mainly in the S and G2/M phases and recruits RNF20 to mitotic centromeres in a centromeric R-loop-dependent manner. In parallel, RPA recruits RNF20 to chromosomal breaks upon DNA damage. Disruption of the RPA–RNF20 interaction or depletion of RNF20 increases mitotic lagging chromosomes and chromosome bridges and impairs BRCA1 and RAD51 loading and homologous recombination repair, leading to elevated chromosome breaks, genome instability, and sensitivities to DNA-damaging agents. Mechanistically, the RPA–RNF20 pathway promotes local H2Bub, H3K4 dimethylation, and subsequent SNF2H recruitment, ensuring proper Aurora B kinase activation at centromeres and efficient loading of repair proteins at DNA breaks. Thus, the RPA–RNF20–SNF2H cascade plays a broad role in preserving genome stability by coupling H2Bub to chromosome segregation and DNA repair.

chromosome segregation | homologous recombination | RPA | H2B ubiquitination | RNF20

Significance

The tumor suppressor RNF20-mediated histone H2B monoubiquitination (H2Bub) plays a critical role in promoting chromosome segregation and DNA repair. However, what is the precise function of RNF20–H2Bub in chromosome segregation and how this pathway is activated to preserve genome stability remain unknown. Here, we identified the role of RNF20–H2Bub in regulating Aurora B kinase activity and chromosome segregation and revealed that it functions via the RNF20–H2Bub–H3K4 dimethylation (H3K4me2)–SNF2H signaling pathway. In parallel, we discovered that the single-strand DNA (ssDNA)-binding protein Replication protein A (RPA) mediates the recruitment of RNF20 to centromeres or chromosomal breaks, thereby coupling H2Bub to chromosome segregation and DNA repair. The broad function of the RPA–RNF20–SNF2H cascade in preserving genome stability makes the pathway an attractive therapeutic target.

Accurate chromosome segregation and DNA repair are essential for preserving genome stability. Aberrant chromosome segregation results in lethality or aneuploidy that leads to abnormal gene dosage and exposes detrimental recessive mutations (1–3). Chromosome missegregation is one of the most common forms of genomic instability and is associated with poor prognosis and therapeutic resistance (4, 5). At the same time, dysfunctions in DNA repair can cause mutations, deletions, insertions, translocation, and even cell death or tumorigenesis (6–8). Therefore, it is crucial to understand how cells precisely regulate chromosome segregation and DNA repair to avoid genome instability, a hallmark and driver of cancer.

For accurate chromosome segregation, kinetochores on the sister chromatids of replicated chromosomes must correctly attach to microtubules from the opposite spindle poles, a process called bi-orientation (2, 9). Aurora B kinase, a component of the conserved chromosomal passenger complex (CPC) that also includes INCENP, Survivin, and Borealin, is a critical player in correcting erroneous kinetochore–microtubule attachments (10–13). Localization of the Aurora B and CPC to centromeres mainly depends on two pathways. In the first, the Haspin kinase phosphorylates histone H3 at Thr3 (H3pT3), which creates a binding site for the CPC component Survivin (14–19). In the second, the Bub1 kinase phosphorylates histone H2A at Thr120 (H2ApT120) to recruit the mitotic protein Shugoshin1, which in turn recruits the CPC, possibly through a linkage to Borealin in human cells (16–18, 20). In early mitosis, high Aurora B kinase activity toward kinetochore substrates inhibits the formation of stable kinetochore–microtubule attachments, while in late mitosis, low activity promotes the stabilization of the attachments (11, 17, 21–24). The Aurora B kinase phosphorylates multiple targets in the conserved outer kinetochore KNL1/Mis12 complex/Ndc80 complex network, a key kinetochore–microtubule attachment interface, to achieve graded levels of microtubule-binding activities (9, 11, 22, 25). As a result, proper localization and activation of Aurora B kinase at centromeres are crucial for accurate chromosome segregation (1, 2, 9, 11, 12).

Faithful repair of chromosomal breaks is essential for cells to pass on their hereditary material intact to the next generation (26). Homologous recombination (HR) is a universal mechanism for the repair of DNA double-stranded breaks (DSBs), the restart of collapsed replication forks, and the generation of gamete cells (27–31). Defects in HR can lead to genome instability and cancer. HR utilizes a homologous sister chromatid as a template

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to direct the repair, and it occurs in the S and G2 phases of the cell cycle (32, 33). During HR, the 5′-ends of a DSB are processed to expose 3′-tailed single-strand DNA (ssDNA). Replication protein A (RPA), a conserved heterotrimeric protein complex composed of RPA1, RPA2, and RPA3, binds to the 3′-end ssDNA tails to activate the DNA damage checkpoint (34, 35). Subsequently, the Rad51 recombinase replaces the RPA bound on ssDNA, initiating DNA pairing and strand invasion of a homologous duplex to form a displacement loop (29, 36, 37). The resolution of the joint molecules accomplishes repair.

Both chromosome segregation and HR repair are regulated by the conserved histone posttranslational modification, H2B monoubiquitination (H2Bub), that occurs on H2B-K120 in human cells (H2B-K123 in budding yeast) (38–43). H2Bub is primarily catalyzed by the E2 ubiquitin conjugase RAD6 or UbcH6 (Rad6 in yeast) in cooperation with the E3 ligase RNF20/RNF40 heterodimer (Bre1 in yeast) (38, 39). Notably, RNF20 functions as a tumor suppressor and alterations of the H2Bub machinery are emerging as ubiquitous features in cancer (44–46).

Human RNF20-mediated H2Bub plays key roles in regulating transcription, centromere integrity, and DNA repair (40–43, 47). RNF20–H2Bub promotes noncoding transcription at centromeres, the lack of which results in chromosome loss, as reflected by increased micronuclei (42, 48). In addition, RNF20–H2Bub promotes DSB repair, likely by enabling the local H3K4 dimethylation (H3K4me2) and subsequent SNF2H recruitment at DSBs (40, 41, 43, 49). However, several key questions regarding the function and regulation of RNF20–H2Bub in chromosome segregation or DNA repair remain unknown. First, what is the precise function and mechanism of RNF20–H2Bub in regulating chromosome segregation? Second, how is the RNF20–H2Bub pathway activated in response to chromosome segregation and DNA repair, and what is the consequence if it is uncoupled from these processes?

In this study, we discovered that RNF20–H2Bub plays an indispensable role in regulating the Aurora B kinase activity and kinetochore–microtubule attachments via the RNF20–H2Bub–H3K4me2–SNF2H/RSF1 pathway during chromosome segregation. Meanwhile, we found that the cell cycle– and DNA damage–dependent RPA–RNF20 interaction couples the RNF20–H2Bub pathway to chromosome segregation or DNA repair via promoting the timely recruitment of RNF20 to centromeres or DSBs. The disruption of the RPA–RNF20 interaction or depletion of RNF20 causes aberrant chromosome segregation and impaired HR, leading to increased chromosome breaks, genome instability, and sensitivities to DNA-damaging agents. Thus, the RPA–RNF20–SNF2H cascade plays a broad role in preserving genome stability by coupling H2Bub to chromosome segregation and DNA repair.

Results

RNF20 Localizes to Centromeres to Promote Local H2Bub and Accurate Chromosome Segregation. To fully characterize the role of RNF20 in human mitotic cells, we first examined its localization at centromeres. We observed that RNF20 costained extensively with the anticentromere antibody (ACA) and Centromere protein C (CENP-C), two markers of inner kinetochores, on mitotic chromosomes of HeLa cells (Fig. 1 *A* and *B* and *SI Appendix*, Fig. S1 *A* and *B*). As expected, the signal of RNF20 was eliminated by stably expressing short hairpin RNAs (shRNA) specifically targeting RNF20 (Fig. 1*B* and *SI Appendix*, Fig. S1*C*). Furthermore, ectopically expressed GFP-RNF20 also colocalized with ACA in prometaphase cells (*SI Appendix*, Fig. S1*D*). Indeed, RNF20 was highly enriched at the centromere region in mitotic cells, as revealed by chromatin immunoprecipitation (ChIP) with multiple primers corresponding to centromeres at different chromosomes (*SI Appendix*, Fig. S1 *E–G*). Next, we carefully assessed the role of RNF20 in maintaining centromere integrity. Knockdown of RNF20 by shRNA in HeLa cells increased the rate of lagging chromosomes from 2.5 to 15% (sevenfold) and chromosome bridges from 2 to 10% (fivefold) (Fig. 1 *C–E*), indicating a defect in whole-chromosome segregation. The defects in the RNF20-depleted cells were largely rescued by reexpressing an shRNA-resistant wild-type RNF20 (RNF20-WT)) (*SI Appendix*, Fig. S2 *A* and *B*). To eliminate the possibility that the phenotype was a consequence of the disruption of RNF20 function in the interphase, we used an auxin-inducible degron (AID) system that allows rapid degradation of the endogenous RNF20 upon the addition of indole-3-acetic acid (IAA) (*SI Appendix*, Fig. S2 *C–G*) (50). As expected, rapid depletion of the mini AID degron-tagged RNF20 in mitosis also significantly increased chromosome missegregation (*SI Appendix*, Fig. S2*H*). Thus, RNF20 plays a direct role in mitosis to promote proper chromosome segregation.

Next, we monitored mitotic progression in cells stably expressing GFP-H2B with time-lapse microscopy. Cells treated with the control shRNA progressed through mitosis in about 50 min on average, while RNF20 depletion prolonged mitotic duration to ~80 min (Fig. 1 *F* and *G* and *SI Appendix*, Fig. S2*I*). Notably, stable RNF20 depletion dramatically increased the levels of micronuclei or polyploidy (*SI Appendix*, Fig. S2 *J* and *K*). Thus, RNF20 is indispensable for timely and accurate chromosome segregation and otherwise leads to chromosome instability.

Next, we tested whether RNF20 functions by promoting H2Bub at centromeres. Indeed, we detected significant enrichment of H2Bub at centromeres in mitotic HeLa cells. As expected, H2Bub enrichment was diminished in RNF20 shRNA cells and was restored upon reexpressing the RNF20-WT (*SI Appendix*, Fig. S3 *A* and *B*). Importantly, overexpression of the H2B-K120R mutant protein, which abolishes H2Bub, led to a similar level of lagging chromosomes or anaphase bridges as seen in RNF20-depleted cells (*SI Appendix*, Fig. S3*C*), suggesting that the mitotic defects in RNF20-depleted cells are attributed to the loss of H2Bub. Together, these results indicate that RNF20 localizes to centromeres, where it catalyzes local H2Bub to ensure proper chromosome segregation.

RNF20-Mediated H2Bub Is Required for Proper Activation of the Aurora B Kinase. Aurora B kinase is a major player in correcting erroneous kinetochore–microtubule attachments that cause lagging chromosomes (10–13, 22, 51, 52). This prompted us to test whether RNF20 affects the localization or activity of Aurora B kinase at centromeres. By immunostaining, we observed that the localization of Thr232-phosphorylated Aurora B (Aurora B-pT232), which is critical for full activation of Aurora B (53), was significantly reduced at centromeres in nocodazole-arrested prometaphase HeLa cells with stable RNF20 depletion (Fig. 2 *A* and *C* and *SI Appendix*, Fig. S4 *A* and *C*). In contrast, the localization of Aurora B or other components of the CPC complex, such as INCENP and Survivin, remained unchanged in RNF20 small interfering RNA (siRNA) cells (Fig. 2 *B* and *C* and *SI Appendix*, Figs. S4 *A–C* and S5 *A–C*). These results suggest that RNF20 depletion specifically impairs the activation rather than the localization of Aurora B at centromeres. Consistently, the global Aurora B-pT232 level was decreased in RNF20-depleted mitotic cells, whereas the Aurora B protein level remained constant (Fig. 2*D*). The reduction in the level of Aurora B-pT232 was restored upon reexpressing the wild-type (WT) *RNF20* allele (*SI Appendix*, Figs. S2 *B* and S4 *D* and *E*). Accordingly, the Aurora B-dependent phosphorylation of histone H3 at Ser10 (pH3S10) was impaired at

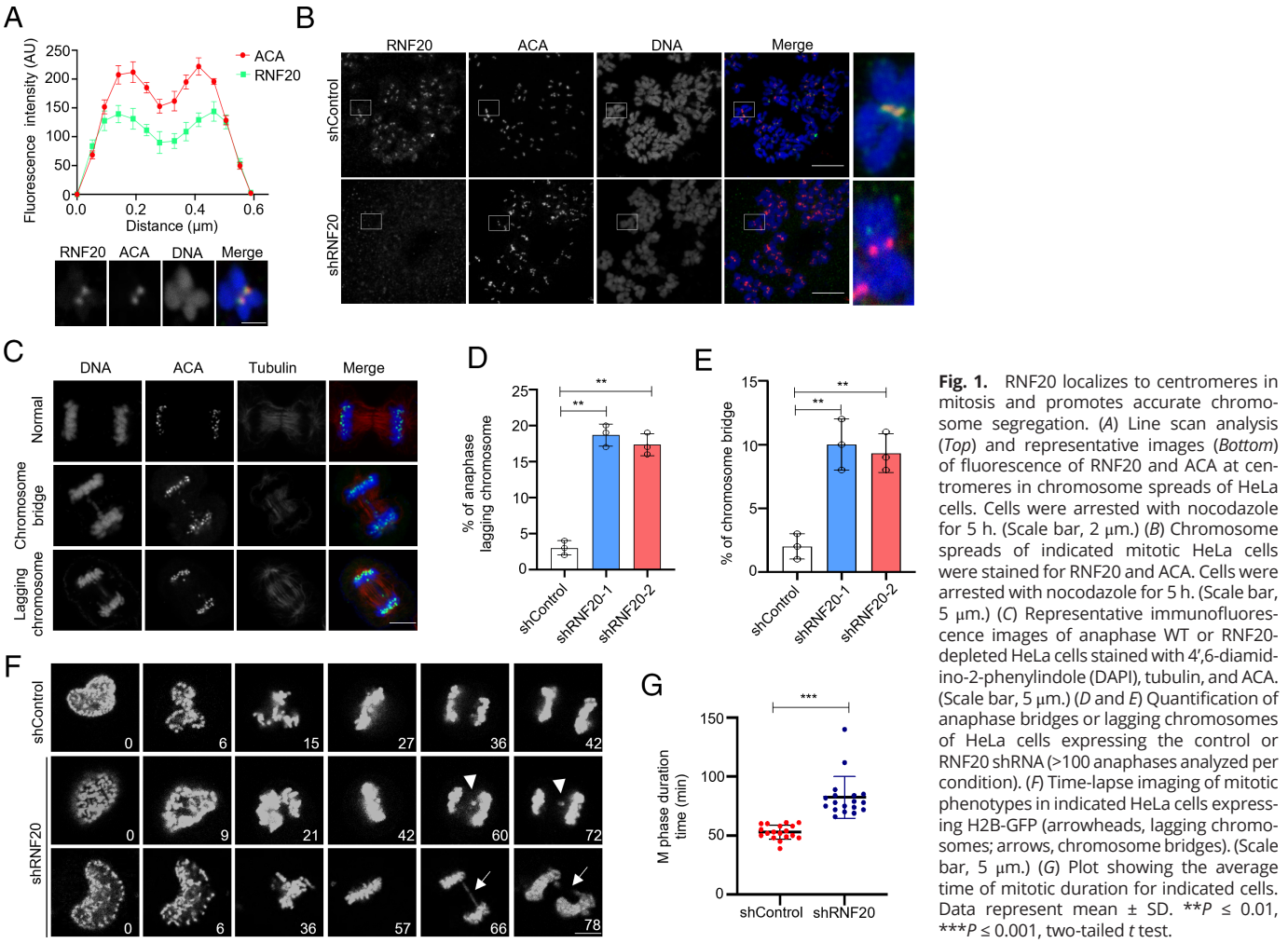

Fig. 1. RNF20 localizes to centromeres in mitosis and promotes accurate chromosome segregation. (*A*) Line scan analysis (*Top*) and representative images (*Bottom*) of fluorescence of RNF20 and ACA at centromeres in chromosome spreads of HeLa cells. Cells were arrested with nocodazole for 5 h. (Scale bar, 2 μm.) (*B*) Chromosome spreads of indicated mitotic HeLa cells were stained for RNF20 and ACA. Cells were arrested with nocodazole for 5 h. (Scale bar, 5 μm.) (*C*) Representative immunofluorescence images of anaphase WT or RNF20-depleted HeLa cells stained with 4′,6-diamidino-2-phenylindole (DAPI), tubulin, and ACA. (Scale bar, 5 μm.) (*D* and *E*) Quantification of anaphase bridges or lagging chromosomes of HeLa cells expressing the control or RNF20 shRNA (>100 anaphases analyzed per condition). (*F*) Time-lapse imaging of mitotic phenotypes in indicated HeLa cells expressing H2B-GFP (arrowheads, lagging chromosomes; arrows, chromosome bridges). (Scale bar, 5 μm.) (*G*) Plot showing the average time of mitotic duration for indicated cells. Data represent mean ± SD. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, two-tailed *t* test.

centromeres in RNF20 siRNA cells (*SI Appendix*, Fig. S6 *A* and *B*). Similarly, when RNF20 was depleted specifically in mitosis using the mAID-degron system, the levels of Aurora B-pT232 and pH3S10 were also reduced (*SI Appendix*, Fig. S6 *C* and *D*).

To test whether RNF20 depletion affects the correction of erroneous kinetochore–microtubule attachments, we performed a monastrol washout assay (54, 55). As expected, we found that the rate of the biorientation defect was significantly increased in RNF20-depleted cells compared to the control (*SI Appendix*, Fig. S5*D*). Consistently, RNF20-depleted cells also failed to undergo accurate chromosome segregation, as elevated lagging chromosomes were observed (*SI Appendix*, Fig. S5*E*). Notably, the global level and centromeric localization of Aurora B-pT232 but not Aurora B were reduced in cells expressing the H2B-K120R mutant protein (Fig. 2 *E*–*G* and *SI Appendix*, Fig. S5*F*). Together, RNF20-mediated H2Bub promotes accurate chromosome segregation, probably via regulating Aurora B kinase activity.

RNF20-Mediated H2Bub Facilitates Centromeric Deposition of H3K4me2 and SNF2H. Next, we explored how RNF20-mediated H2Bub may affect Aurora B-pT232 level and chromosome segregation. At transcribed chromatin, H2Bub is required for di- and tri-methylation of H3K4 and H3K79 (40, 41, 56). At DSBs, H2Bub facilitates local H3K4me2, which subsequently recruits the chromatin remodeler SNF2H to remodel chromatin structures (41, 49). To test whether RNF20 acts via the same mechanism at centromeres, we analyzed the centromeric deposition of H3K4me2 and SNF2H by ChIP. Like H2Bub, both H3K4me2 and SNF2H were highly enriched at the core centromere region. Notably, the depletion of RNF20 significantly impaired their centromeric deposition, and this defect was largely rescued by reexpressing an shRNA-resistant WT *RNF20* allele (Fig. 2 *H* and *I* and *SI Appendix*, Fig. S7). Thus, RNF20-mediated H2Bub promotes H3K4me2 deposition and subsequent SNF2H recruitment at centromeres. Notably, the depletion of SNF2H by siRNA increased the rate of lagging chromosomes and anaphase bridges compared to the control (Fig. 2*J*). Importantly, depletion of SNF2H alone and concurrent depletion of SNF2H and RNF20 caused a similar extent of mitotic aberration phenotypes (Fig. 2*K* and *SI Appendix*, Fig. S8*A*), indicating that RNF20 and SNF2H act in a common pathway to ensure proper chromosome segregation.

The RNF20–H2Bub–H3K4me2–SNF2H Cascade Controls RSF1 Level and Aurora B Activity. SNF2H, together with RSF1, forms the remodeling and spacing factor (RSF) complex that plays a critical role in regulating nucleosome assembly and repositioning (49, 57, 58). Interestingly, SNF2H is required to maintain the RSF1 protein level (59, 60). Furthermore, previous studies reported that RSF1 is required for faithful chromosome segregation by facilitating PLK1 recruitment and, thereby, the activation of Aurora B kinase (59). We thus reasoned that the RNF20–SNF2H axis might regulate chromosome segregation by controlling the centromeric localization of RSF1 and the downstream Aurora B kinase activity. As expected, the depletion of either RNF20 or SNF2H by siRNAs impaired the

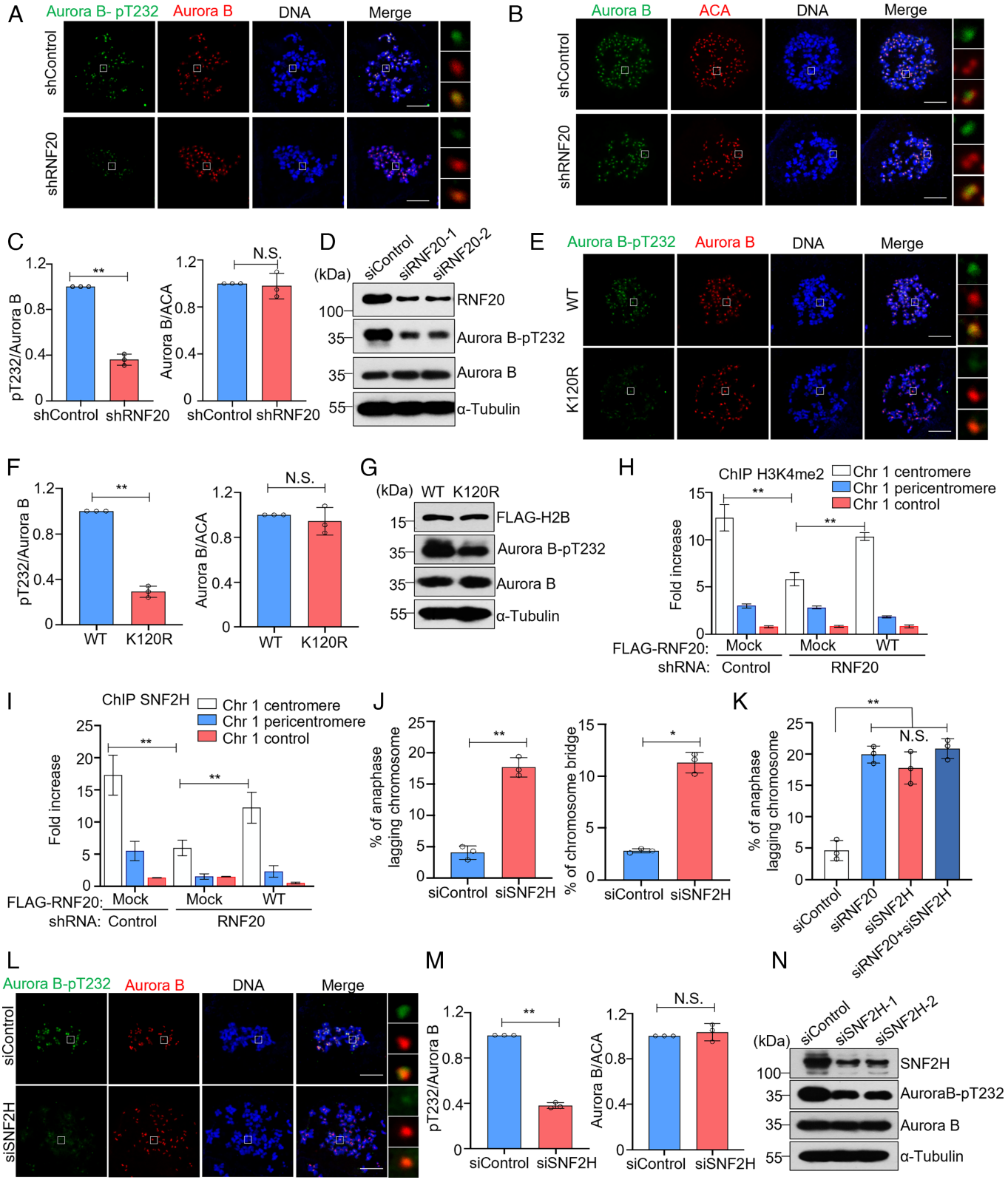

Fig. 2. RNF20-mediated H2Bub is required for proper activation of the Aurora B kinase. (*A–C*) Fluorescence intensity and quantification of Aurora B-pT232 and Aurora B at centromeres in prometaphase HeLa cells expressing the control or RNF20 shRNA. Mitotic cells were obtained after nocodazole treatment for 5 h. (Scale bar, 5 μm.) (*D*, *G*, and *N*) Immunoblotting analysis of indicated proteins in HeLa cells transfected with the control, RNF20 or SNF2H siRNA, or the plasmid overexpressing the FLAG-H2B or FLAG-H2B-K120R protein. Mitotic cells were obtained after nocodazole treatment for 16 h. (*E* and *F*) Fluorescence intensity and quantification of Aurora B-pT232 at centromeres in prometaphase or metaphase HeLa cells overexpressing the FLAG-H2B or FLAG-H2B-K120R protein. (Scale bar, 5 μm.) (*H* and *I*) ChIP-qPCR showing centromeric deposition of H3K4me2 or SNF2H in indicated mitotic HeLa cells. RNF20 shRNA cells were transfected with an empty vector or a plasmid expressing FLAG-RNF20. Mitotic cells were obtained after nocodazole treatment for 16 h. (*J* and *K*) Percentage of anaphases from indicated mitotic cells that display lagging chromosomes or anaphase bridges. (*L* and *M*) Fluorescence intensity and quantification of Aurora B-pT232 at centromeres in mitotic HeLa cells expressing the control or SNF2H siRNA. Mitotic cells were obtained after nocodazole treatment for 5h. (Scale bar, 5 μm.) Error bars in all panels represent SD. **P* ≤ 0.05, ***P* ≤ 0.01 two-tailed *t* test.

centromeric localization of RSF1 in mitotic HeLa cells (*SI Appendix*, Fig. S8 *B* and *C*). In addition, the lack of SNF2H attenuated the centromeric localization or total levels of Aurora B-pT232 but not Aurora B itself (Fig. 2 *L–N* and *SI Appendix*, Fig. S8*D*), as observed in RSF1 siRNA cells (59). Together, these results indicate that RNF20-dependent H2Bub regulates chromosome segregation by promoting the centromeric deposition of H3K4me2 and SNF2H, which further allows RSF1 recruitment and Aurora B activation.

RNF20/RNF40 Interacts with the RPA Complex In Vivo and In Vitro. We then asked how RNF20 was recruited to centromeres. Our previous work in yeast demonstrated that RPA recruits Bre1, the yeast homolog of RNF20, to replication forks and DNA breaks to promote DNA replication and repair (61). Therefore, we tested whether RPA recruits RNF20 to centromeres in human cells. We found that ectopically expressed FLAG-tagged RNF20 or RNF40 coimmunoprecipitated with Myc-tagged RPA1, RPA2, and RPA3, but not the Morc3 control protein (Fig. 3*A* and *SI Appendix*, Fig. S9*A*). Consistently, the endogenous RNF20/40 and RPA can mutually precipitate each other in human HCT116 or HeLa cells (Fig. 3 *B* and *C*). Notably, this interaction occurs primarily in the S and G2/M phases (Fig. 3*D*).

Next, we incubated purified glutathione-S-transferase (GST)-tagged RNF20 or RNF40 with 6xHis-tagged RPA1, RPA2, or RPA3 individually and performed pull-down assays. We observed that GST-RNF20 or RNF40, but not GST itself, directly interacted with RPA1 and RPA2 (Fig. 3*E*). To identify the RNF20 motif that mediates the interaction with RPA, we expressed a series of FLAG-tagged RNF20 truncations in HEK293T cells and examined their interactions with endogenous RPA by immunoprecipitation (Fig. 3*F*). Compared to the WT RNF20, the RNF20-D6 truncation, which lacks the residues from 590 to 703, exhibited a much weaker interaction with RPA (Fig. 3 *F* and *G*). Indeed, this fragment (590 to 703 aa) directly interacts with RPA1 or RPA2 in vitro (*SI Appendix*, Fig. S9*B*). Within this fragment, we identified an evolutionarily conserved motif (678 to 693 aa) that is also shared by several other RPA2-binding proteins (62) (*SI Appendix*, Fig. S9*C*). Next, we turned to delineate the motifs in RPA1 and RPA2 that mediate the interaction with RNF20. We found that the N-terminal ends of RPA1 (1 to 110 aa) and RPA2 (1 to 45 aa) are essential for mediating the interaction with RNF20 (*SI Appendix*, Fig. S9 *D–G*). Thus, we demonstrated the direct interaction between RNF20/40 and RPA and defined the domains mediating their associations.

The RPA-Dependent RNF20 Loading at Centromeres Ensures Proper H2Bub and Chromosome Segregation. We examined the possible role of the RPA–RNF20 interaction in regulating chromosome segregation. As previously reported, we observed that RPA localized to centromeres in mitotic HeLa cells (*SI Appendix*, Fig. S10) (63). However, the knockdown of RPA1 with siRNA impaired RNF20 localization at centromeres (Fig. 4*A*), implying that RPA is required for the centromeric deposition of RNF20. Consistently, the ectopically expressed FLAG-tagged WT RNF20 properly localized to centromeres, while the RNF20-D6 mutant protein, which cannot bind RPA, failed to do so (Fig. 4*B*), suggesting that RPA directly recruits RNF20 to centromeres. Accordingly, specific disruption of the RPA–RNF20 interaction by reexpressing the RNF20-D6 mutant protein in RNF20 shRNA cells impaired the deposition of H2Bub, H3K4me2, and SNF2H at centromeres, as compared to cells reexpressing WT RNF20 (Fig. 4 *C–E* and *SI Appendix*, Figs. S3*A* and S7). Consequently, the aberrant mitotic defects resulting from RNF20 depletion were suppressed by reexpressing the WT RNF20 but not the RNF20-D6 mutant protein (Fig. 4*F*).

To exclude the possibility that the RNF20-D6 truncation may affect its E3 ligase activity, we cotransfected HEK293T cells with plasmids overexpressing HA-eEF1BδL, His-ubiquitin, RNF40, and FLAG-RNF20 or RNF20-D6. We observed that eEF1BδL, a known target of RNF20/40 (64), was efficiently ubiquitinated by both RNF20 and RNF20-D6 proteins (*SI Appendix*, Fig. S11). As a control, its ubiquitination was impaired in cells overexpressing an RNF20 lacking the RING domain (RNF20-RINGΔ) (*SI Appendix*, Fig. S11). These results suggest that the RNF20-D6 mutant protein has normal ligase activities. Thus, we conclude that RPA targets RNF20 to centromeres in mitosis to promote the local deposition of H2Bub, H3K4me2, and SNF2H and to ensure accurate chromosome segregation.

Centromeric R-Loops Are Required for RNF20 Deposition at Centromeres. In mitosis, RPA is recruited to centromeres by centromeric R-loops to activate ATR kinase, which allows the subsequent activation of CHK1 and Aurora B kinases to ensure proper chromosome segregation (63). Therefore, we tested whether the presence of R-loops is required for RNF20 centromeric deposition. Indeed, we detected R-loops at centromeres by DNA–RNA immunoprecipitations (DRIP) using the S9.6 antibody that recognizes DNA–RNA hybrids (*SI Appendix*, Fig. S12*A*). To exclude the potential nonspecificity of the S9.6 antibody (65, 66), we performed DRIP using the V5-tagged RNaseH1 mutant ($RNaseH1^{D210N}$) that is RNase-dead so that it can bind but does not cleave DNA–RNA hybrids. Consistently, we detected a robust enrichment of DNA–RNA hybrids at the centromere. However, the signal was significantly reduced when pretreating the samples with RNase H prior to immunoprecipitation (*SI Appendix*, Fig. S12*B*). Accordingly, overexpression of $RNaseH1^{WT}$ greatly reduced the enrichment of R-loops and, subsequently, RNF20 at centromeres, while overexpression of the $RNaseH1^{D210N}$ mutant did not impair their centromeric enrichment (Fig. 4*G* and *SI Appendix*, Fig. S12*A*) (67). Consistently, the colocalization of RPA or RNF20 with ACA and the activation of Aurora B were impaired upon the expression of $RNaseH1^{WT}$ but not $RNaseH1^{D210N}$ (Fig. 4*H* and *SI Appendix*, Fig. S12 *C* and *D*). Thus, the R-loop-dependent RPA localization is critical for centromeric deposition of RNF20 and Aurora B activation. Notably, the depletion of RNF20 did not impair the activation of ATR, as indicated by the phosphorylation of ATR on Thr1989 (*SI Appendix*, Fig. S13) (68, 69), suggesting that the RPA-dependent RNF20 deposition at centromeres functions independent of the role of RPA in activating ATR. Together, our results establish that the RPA–RNF20–H2Bub–H3K4me2–SNF2H (RPA–RNF20–SNF2H) cascade plays an important role in promoting Aurora B kinase activation and timely and accurate chromosome segregation.

RPA Recruits RNF20 to DSBs in a Manner Dependent on ATM/ATR Activities. Several previous studies have implicated the role of RNF20 in promoting DSB repair (40, 41, 43). RNF20–H2Bub appears to function by enabling local H3K4me2 and subsequent SNF2H recruitment, which alters the local chromatin structures and permits the access of repair proteins (41, 49). However, how the RNF20–H2Bub pathway is activated in response to DNA damage is unknown. First, we tested whether the RPA–RNF20 interaction is required for RNF20 recruitment at DSBs. As expected, RNF20 formed stripes or foci that colocalize with γH2AX following UV microirradiation or etoposide (VP16) treatment (Fig. 5*A* and *SI Appendix*, Fig. S14*A*). Consistently, RNF20 is recruited to the I-*Sce*I-induced DSB ends (*SI Appendix*, Fig. S14*B*). Notably, we found that RNF20 colocalized with RPA after DNA damage (Fig. 5 *A* and *B*). However, the depletion of

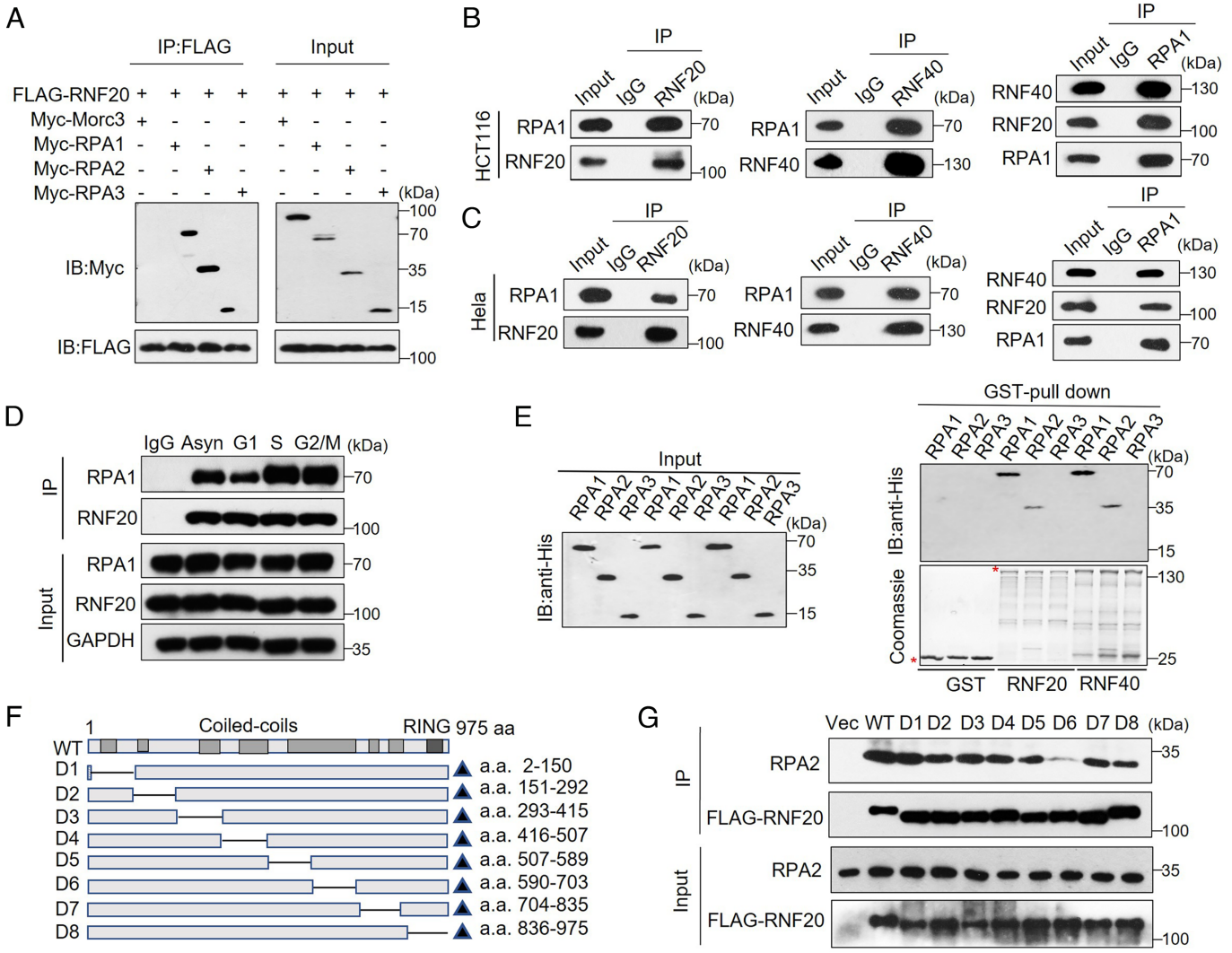

Fig. 3. RNF20/RNF40 interacts with the RPA complex in vivo and in vitro. (*A*) RNF20 interacts with RPA but not the Morc3 control protein. HEK293T cells were transiently transfected with a plasmid expressing FLAG-tagged RNF20 and a plasmid encoding either the Myc-tagged RPA1, RPA2, RPA3, or Morc3. Immunoprecipitation and western blot were performed with indicated antibodies. (*B–D*) Immunoprecipitations showing the interactions between endogenous RNF20/40 and RPA in indicated cell lines or cell cycle stages. (*E*) GST pull-down assay showing direct interactions between the recombinant GST-RNF20 or GST-RNF40 and 6xHis-tagged RPA1, RPA2, or RPA3. GST serves as a negative control. * indicates the full-length GST-RNF20 or GST-RNF40 protein. (*F*) Scheme showing the full-length and truncated RNF20 proteins. Dash lines indicate truncated regions. Coiled coils and the RING domain are indicated. (*G*) Immunoprecipitation showing the interaction between endogenous RPA and ectopically expressed FLAG-tagged WT or truncated RNF20 in HEK293T cells.

RPA1 severely impaired RNF20 foci formation, yet it did not change the protein level of RNF20 or RNF40 (Fig. 5 *B* and *C* and *SI Appendix*, Fig. S14*C*). Importantly, ectopically expressed FLAG-tagged WT RNF20 but not the RNF20-D6 mutant protein appropriately localized to sites of DNA damage (Fig. 5 *D–F*), suggesting that the RPA–RNF20 interaction is important for the localization of RNF20 at DNA breaks. To validate this result, we measured the assembly of GFP-RNF20 on the laser stripes in cells receiving siRNA against RPA1. Again, we observed that RNF20 accumulation at DNA damage sites was reduced in RPA1-depleted cells, which was rescued by the expression of a siRNA-resistant WT RPA1 (Fig. 5*G*). Together, these results demonstrate that RNF20 is recruited to DSBs through its physical interaction with RPA.

Accordingly, the interaction between RNF20 and RPA was markedly increased in HeLa or HCT116 cells following hydroxyurea (HU), camptothecin (CPT), or VP16 treatment (Fig. 5*H*), suggesting that the DNA damage signal stimulates the interaction. Indeed, exposure of HCT116 cells to the Ataxia telangiectasia mutated (ATM) or ataxia telangiectasia and Rad3-related (ATR) kinase inhibitor after VP16 treatment mildly attenuated the interaction, while simultaneous inhibition of both kinases dramatically impaired the association, suggesting that ATM and ATR redundantly regulate this interaction (Fig. 5*I* and *SI Appendix*, Fig. S14*D*). Thus, the DNA damage-induced ATM/ATR-dependent checkpoint signal stimulates the RPA–RNF20 interaction that further facilitates RNF20 recruitment at DSB ends.

The RPA–RNF20–SNF2H Cascade Promotes Efficient HR Repair. As expected, we detected robust H2Bub enrichment at the I-*Sce*I-induced DSB ends, and this enrichment disappeared upon RNF20 depletion (Fig. 6*A*). This defect was fully rescued by reexpressing a siRNA-resistant WT *RNF20*. However, it can only be partially restored by reexpressing the *RNF20-D6* mutant allele (Fig. 6*A*). Thus, the RPA–RNF20 interaction is required for proper H2Bub at DSBs.

Next, we assessed the role of the RPA–RNF20 interaction in DNA damage repair. As shown in Fig. 6*B* and *SI Appendix*, Fig. S15, RNF20-depleted HeLa cells accumulated more

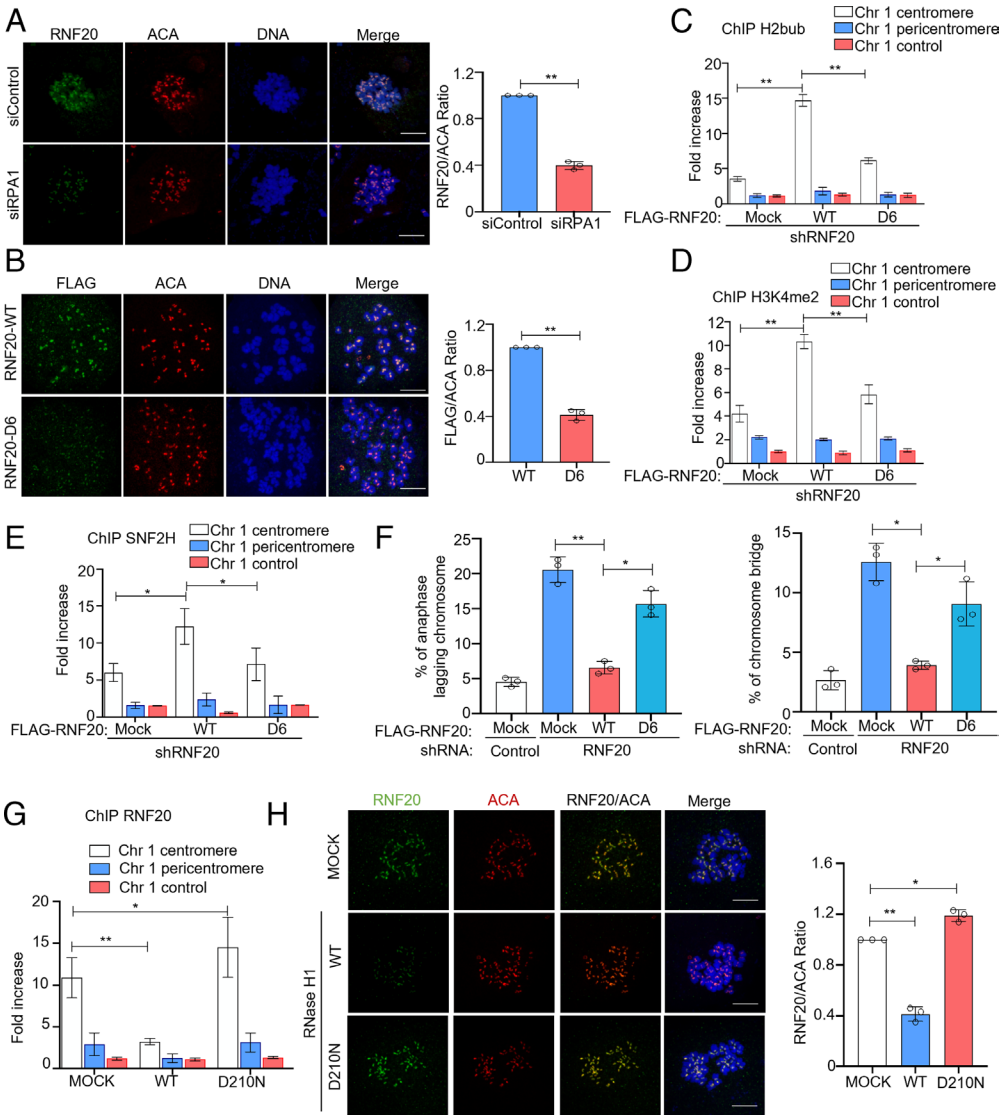

Fig. 4. RPA-dependent RNF20 deposition is required for centromeric H2Bub and accurate chromosome segregation. (*A*) Fluorescence intensity and quantification of RNF20 localization at centromeres in prometaphase HeLa cells expressing the control or RPA1 siRNA. Mitotic cells were obtained after nocodazole treatment for 5 h prior to chromosome spread immunostaining. The graph represents the relative intensity of RNF20 against ACA at kinetochores. (Scale bar, 5 μm.) (*B*) Fluorescence intensity of the FLAG-tagged WT or D6 truncation RNF20 at centromeres in mitotic HeLa cells expressing shRNA against RNF20. RNF20-depleted cells were introduced with indicated RNF20 constructs and treated with nocodazole for 5 h prior to chromosome spread immunostaining. The graph represents the relative intensity of RNF20 against ACA at kinetochores. (Scale bar, 5 μm.) (*C–E*) ChIP-qPCR analysis of H2Bub, H3K4m2, and SNF2H enrichment in mitotic RNF20 shRNA HeLa cells expressing an empty vector or the shRNA-resistant WT or D6 truncation RNF20 construct. (*F*) Percentage of anaphases in shRNF20 HeLa cells with anaphase bridges or lagging chromosomes after introducing the plasmid expressing the WT or D6 truncation RNF20. (*G*) ChIP-qPCR analysis of RNF20 enrichment in mitotic HeLa cells with plasmids expressing the WT or D210N mutant RNaseH1. (*H*) Fluorescence intensity and quantification of RNF20 localization at centromeres in prometaphase HeLa cells expressing the WT or D210N mutant RNaseH1. (Scale bar, 5 μm.) Error bars in this figure represent SD. **P* ≤ 0.05, ***P*, ≤ 0.01 two-tailed *t* test.

spontaneous γH2AX. Importantly, after transient VP16 treatment, RNF20 shRNA cells exhibited slower kinetics in the removal of γH2AX compared to the control, implying that these cells are defective in repairing DNA damage. Using the DR-GFP reporter system, we confirmed that the HR repair was compromised in RNF20-depleted cells (Fig. 6*C*) (40, 41, 43). Consistently, RNF20 depletion impaired BRCA1 and RAD51 recruitment at DSBs and compromised cell survival upon CPT or VP16 treatment (Fig. 6 *D–G*). Notably, all these defects were fully rescued by reexpressing an shRNA-resistant WT *RNF20* but not by the *RNF20-D6* mutant allele (Fig. 6 *B–G* and *SI Appendix*, Fig. S15). Thus, the RPA-dependent RNF20 recruitment at DSBs is important to promote H2Bub, BRCA1 and RAD51 recruitment, HR repair, and DNA damage response. Together with previous studies, we propose that the RPA–RNF20–H2Bub–H3K4me2–SNF2H cascade plays an indispensable role in promoting DSB repair by HR.

Reduction of RNF20 Causes Chromosomal Breaks and Correlates with Genome Instability. In line with the roles of RNF20 in chromosome segregation and HR repair, we observed that the RNF20-deficient HeLa or MCF7 cells exhibited a higher rate of chromosomal/chromatid breaks no matter with or without CPT treatment (Fig. 7 *A* and *B*). Moreover, the loss of RNF20 caused elevated sensitivity to the PARP inhibitor olaparib in both cell lines (Fig. 7 *C* and *D*). By analyzing breast cancer data from the TCGA database, we found that RNF20 mRNA levels are reduced in tumors, and low RNF20 levels significantly correlate with poor survival of breast cancer patients (Fig. 7 *E* and *F*). Furthermore, the high levels of chromatin instability represented by telomeric allelic imbalances, loss of heterozygous (LOH), and the large-scale transition significantly correlated with the low expression of RNF20 in breast cancer (Fig. 7*G*). Thus, proper expression of RNF20 is important to suppress tumorigenesis and chromosomal instability. Together, our results reveal a broad and indispensable function of the RPA–RNF20–SNF2H cascade in ensuring accurate chromosome segregation and efficient HR repair.

Discussion

Accurate chromosome segregation and DNA repair are crucial for the maintenance of genome stability. In this study, we identified the precise function and mechanism of RNF20–H2Bub in promoting chromosome segregation and revealed how the RNF20–H2Bub pathway is evoked in response to chromosome segregation and DNA repair. We show that the RPA–RNF20–SNF2H cascade plays a key role in these processes. We found that RPA interacts with RNF20 in a cell cycle–dependent manner, and the centromeric R-loop-dependent localization of RPA recruits the tumor

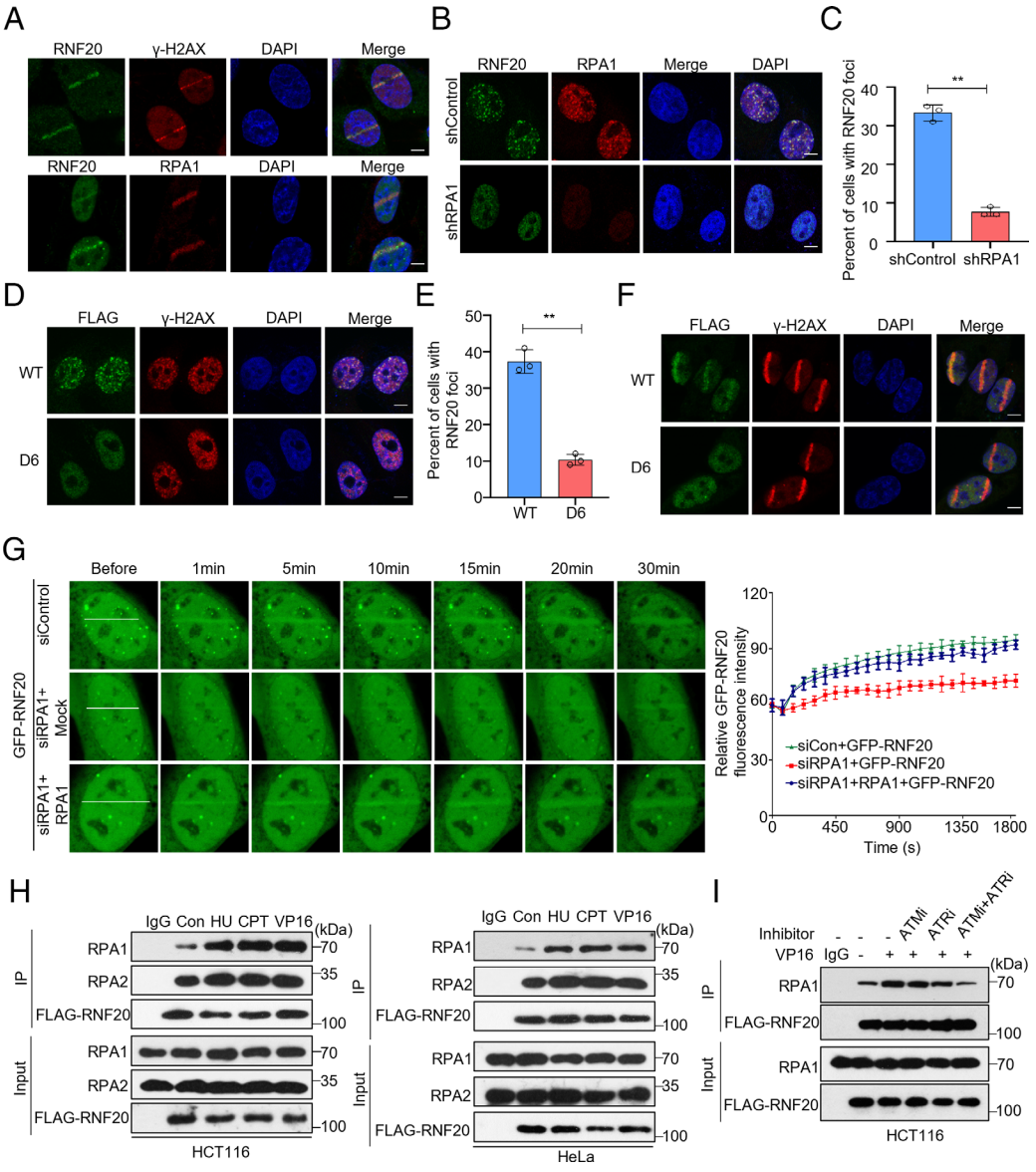

Fig. 5. RPA recruits RNF20/40 to the sites of DNA lesion. (*A*) Immunostaining showing RNF20 colocalization with γH2AX or RPA upon laser microirradiation in HeLa cells. Cells transfected with FLAG-tagged RNF20 were irradiated. After 1 h, the cells were stained with anti-FLAG or anti-γH2AX antibody. (Scale bar, 5 μm.) (*B* and *C*) Immunostaining and quantification showing the colocalization of RNF20 with RPA in the control or RPA1-depleted HeLa cells. (Scale bar, 5 μm.) (*D–F*) Immunostaining showing the colocalization of RNF20 with γH2AX in RNF20 shRNA HeLa cells expressing the WT or D6 truncation RNF20. The cells were treated with VP16 for 1 h or recovered for 1 h post laser microirradiated. (Scale bar, 5 μm.) Quantification of the signal in (*D*) is presented in *E*. (*G*) Live cell imaging showing GFP-RNF20 recruitment in U2OS cells transfected with the control or RPA1 siRNA, followed by complementation with an empty vector or a plasmid expressing the siRNA-resistant RPA1. After 48 h, the cells were subjected to laser microirradiation. The intensity of each laser stripe at each time point was determined by averaging values from over 20 cells. (*H* and *I*) Immunoprecipitation showing the RPA–RNF20 interaction after treatment with different DNA-damaging agents or/and inhibitors against ATM or ATR. Cells transfected with FLAG-RNF20 were cultured for 48 h followed by HU, CPT, and VP16 treatment. The small-molecule inhibitors specific for ATM (KU55933, 10 μM) and ATR (VE-821, 10 μM) were added 2 h before DNA damage treatment. The anti-FLAG immunoprecipitates were analyzed with indicated antibodies. Error bars denote in this figure represent SD. $^{**}P \leq 0.01$, two-tailed *t* test.

suppressor RNF20 to centromeres in mitotic cells (Figs. 1, 3, and 4). The centromeric deposition of RNF20 stimulates local H2Bub, H3K4me2, and subsequent SNF2H recruitment (Figs. 2 and 4), which further promotes RSF1 recruitment and the activation of Aurora B kinase that regulates erroneous kinetochore–microtubule attachments (Fig. 2 and *SI Appendix*, Fig. S8 *B–D*), thus ensuring proper chromosome segregation (Fig. 4*F* and *SI Appendix*, Fig. S16). Thus, we discovered a specific function of RNF20–H2Bub in controlling Aurora B kinase activity and kinetochore–microtubule attachments. In parallel, we showed that RPA interacts with RNF20 in a DNA damage–dependent manner, which facilitates the recruitment of RNF20 by RPA to chromosomal breaks to promote DNA damage response and HR repair (Figs. 5 and 6). Together with the previously established role of RNF20 in DSB repair (41, 49), we conclude that the RPA–RNF20–SNF2H cascade also functions at DSBs to promote HR repair (*SI Appendix*, Fig. S16). Consequently, disruption of the RPA–RNF20–SNF2H cascade causes defects in chromosome segregation and HR repair, leading to genome instability and susceptibility to DNA-damaging agents (Fig. 7).

In summary, the RPA–RNF20–SNF2H cascade plays two layers of function in preserving genome stability. First, the regulated RPA–RNF20 interaction couples the RNF20–H2Bub pathway to chromosome segregation or DNA repair by facilitating the timely recruitment of RNF20 to centromeres or DSBs. Second, the RNF20–H2Bub–H3K4me2–SNF2H pathway promotes the activation of Aurora B kinase activity and efficient loading of HR proteins, thereby ensuring accurate chromosome segregation and efficient DNA repair.

RPA Recruits RNF20 to Centromeres and DSBs. It was unknown how RNF20 is timely and specifically targeted to centromeres or chromosomal breaks. Although RNF20 was shown to interact with NBS1 or PAF1, the depletion of NBS1 or PAF1 does not affect RNF20 recruitment at DSBs (41, 70). Here, we found that RPA directly interacts with RNF20 and recruits the enzyme to centromeres and DSBs (Figs. 4 and 5). Depletion of RPA or specific disruption of their interaction impairs RNF20 loading, leading to improper chromosome segregation and HR repair (Figs. 4 and 6). Interestingly, our previous work revealed that RPA recruits Bre1, the yeast homolog of RNF20, to replication forks and DSBs to promote DNA replication and repair (61). Thus, RPA-mediated RNF20 recruitment is likely a conserved mechanism across species.

On the contrary, it appears that RNF20 could also facilitate the initial loading of RPA. For example, RNF20 localization at DSBs is

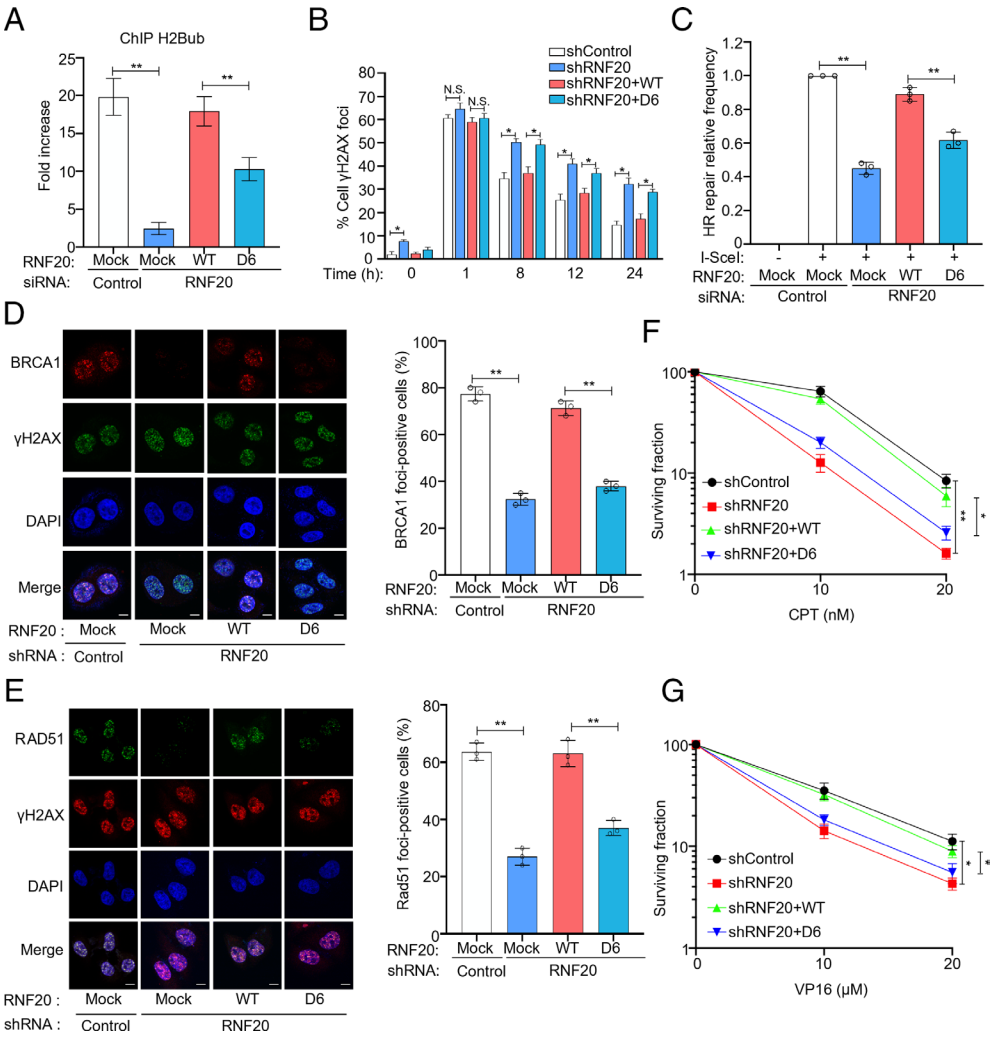

Fig. 6. RPA recruits RNF20 to DSBs to promote local H2B ubiquitination and homologous recombination repair. (*A*) ChIP-qPCR analysis of H2Bub enrichment in indicated cells. The DR-GFP reporter U2OS cells were transfected with indicated siRNAs or combination of siRNA and siRNA-resistant WT or D6 truncation RNF20 construct. The cells were then transfected with or without an I-*Sce*I-expressing plasmid for 24 h. (*B*) Quantification of γH2AX foci number along the recovery post VP16 treatment in indicated cells. A plasmid expressing the FLAG-tagged WT or D6 truncation RNF20 was transfected into shRNF20 HeLa cells. The cells were then treated with 5 μM VP16 for 1 h and recovered for various time points before labeling with an anti-γH2AX antibody. Quantification results from the average of three independent experiments are shown as mean ± SD. More than 100 cells were counted in each group. (*C*) Comparison of HR repair capacity in DR-GFP U2OS cells transfected with indicated siRNAs or a combination of siRNA and plasmid expressing a siRNA-resistant WT or D6 mutant RNF20 protein. (*D* and *E*) Immunofluorescent analysis of BRCA1 or RAD51 foci formation in indicated cells upon DNA damages. HeLa cells stably expressing shRNA-targeting RNF20 were complemented with a plasmid expressing siRNA-resistant WT or D6 truncation RNF20. After 48 h, the cells were treated with 5 μM VP16 for 1 h, followed by staining with anti-BRCA1 (*D*), RAD51 (*E*), or γH2AX antibodies. DAPI was used as an indicator for the nucleus. The percentage of cells with positive BRCA1 or Rad51 foci from >100 counts was determined. (Scale bar, 5 μm.) (*F* and *G*) Survival curves for indicated cells treated with different concentrations of CPT or VP16. Error bars in this figure represent SD. $*P \le 0.05$, $**P \le 0.01$, two-tailed *t* test.

required for proper loading of BRCA1, which is known to promote DNA end resection and subsequent RPA loading (71). This appears to be contradictory to the conclusion that RPA recruits RNF20 at DSBs. We consider that RNF20–SNF2H-mediated chromatin relaxation and subsequent DNA end resection permit the initial loading of RPA, while the binding of RPA to ssDNA activates the DNA damage checkpoint, which in turn stimulates the RPA–RNF20 interaction and further promotes the extensive recruitment and spreading of RNF20 along chromatin. Similarly, the RNF20-dependent transcription might stimulate the formation of R-loops at centromeres, which allows the initial loading of RPA. In the S and G2/M phases, the enhanced RPA–RNF20 interaction acts in turn to stimulate RNF20 deposition at centromeres to ensure localized transcription.

Previous work showed that RPA functions by activating ATR at centromeres, which subsequently targets CHK1 to activate Aurora B kinase, thereby ensuring proper chromosome segregation (63). Here, we found that RNF20 depletion does not affect ATR activation at centromeres (*SI Appendix*, Fig. S13), suggesting that the RPA–RNF20–SNF2H cascade acts independently of the established RPA–CHK1 pathway in regulating Aurora B kinase activity. Thus, RPA exerts two layers of functions at centromeres.

The Cell Cycle–Dependent Centromeric Transcription and RPA–RNF20 Interaction. RNF20–H2Bub enrichment and transcription at centromeres are controlled by the cell cycle and increase specifically before the onset of the M phase (42). Centromeric transcription is important for the proper localization of CENP-A and CENP-C and the assembly of the kinetochore (72–76). In addition, centromeric RNAs could stimulate Aurora B kinase activity (48, 77). Here, we found that the RNF20–H2Bub cascade could facilitate local recruitment of SNF2H/RSF1, providing a unique mechanism of how centromeric transcription might contribute to chromosome segregation.

However, how these events are controlled by the cell cycle remains unknown. We found that the RPA–RNF20 interaction primarily occurs in the S and G2/M phases (Fig. 3*D*). This cell cycle–dependent interaction is likely critical to restrain RNF20–H2Bub deposition and centromeric transcription to the G2/M phases. Interestingly, the RPA–RNF20 interaction is also enhanced upon the DNA damage in the ATM- and ATR-dependent manner (Fig. 5 *H* and *I*). We envision that the cell cycle– or DNA damage–dependent regulation of the RPA–RNF20 interaction might play an important role in defining the chromatin landscape of H2Bub, thus ensuring the spatiotemporal control of DNA replication, repair, and chromosome segregation and avoiding their unscheduled conflicts with transcription. However, how CDKs and ATM/ATR precisely control the RPA–RNF20 interaction awaits further investigations. We speculate that the CDK-dependent or the DNA damage-induced phosphorylation of RPA or RNF20 likely regulates their interaction.

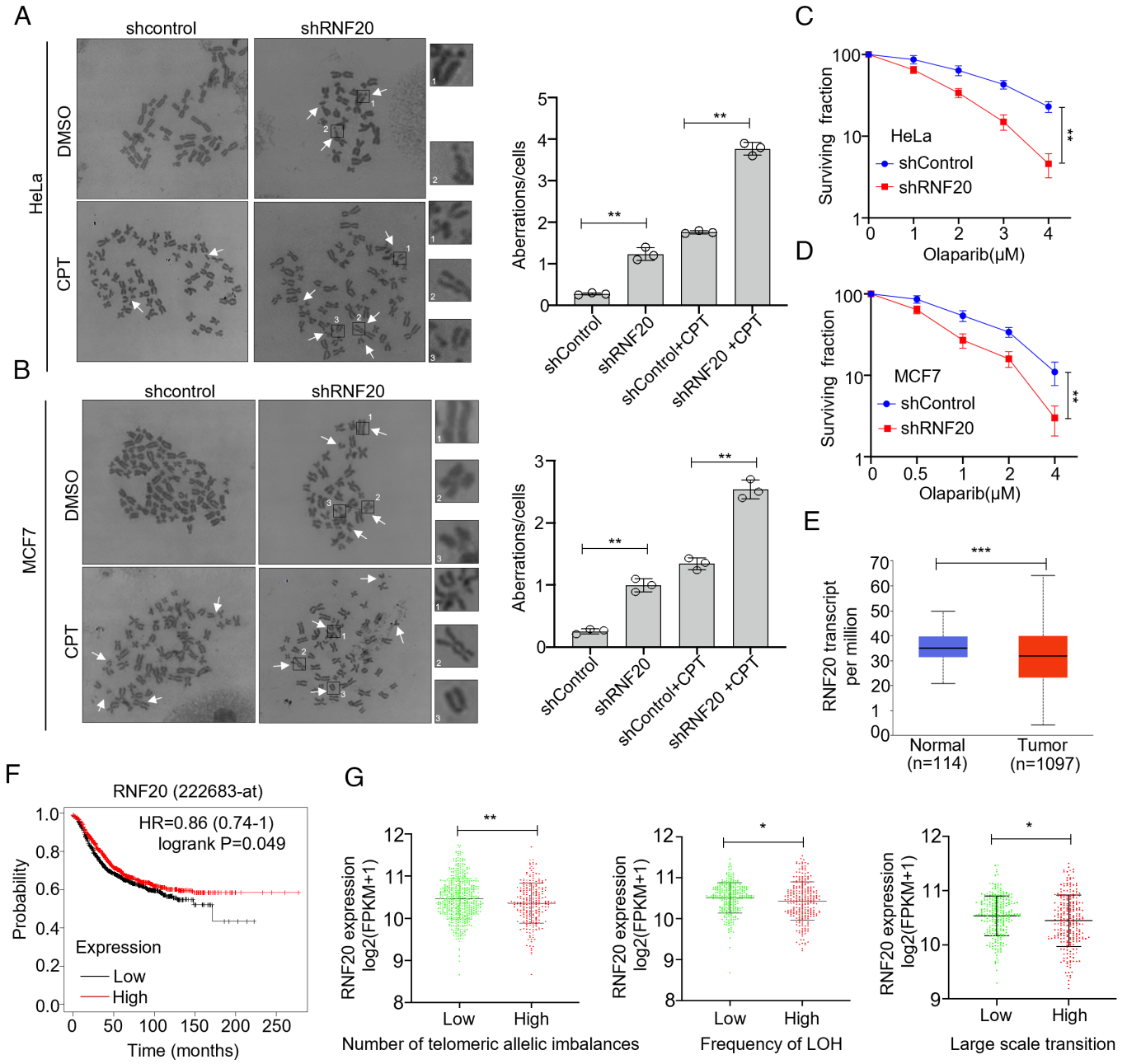

Fig. 7. RNF20 suppresses chromosomal breaks and genomic instability. (*A* and *B*) Chromosomal spreading analysis of metaphase chromosomal breaks in the WT or RNF20-deficient HeLa or MCF7 cells. Cells were either treated with dimethyl sulfoxide (DMSO) or 40 μM CPT for 12 h before analysis. Chromosomal breaks are indicated by arrows. Quantification results are presented in the graphs. More than 50 mitotic chromosomes were randomly analyzed. (*C* and *D*) Sensitivity of WT or RNF20-deficient HeLa or MCF7 cells to olaparib. (*E*) Boxplots showing the mRNA expression level of RNF20 in normal and invasive breast carcinoma (http://ualcan.path.uab.edu/). (*F*) Kaplan–Meier overall survival analysis of RNF20 mRNA expression in 3,458 breast cancer patients from a public dataset (http://kmplot.com/analysis/index.php?p=service&cancer=breast). (*G*) Correlation between RNF20 expression levels and chromosomal instability, including frequency of LOH, large-scale transition, and the number of telomeric allelic imbalances. The graphs represent means ± SD. **P* ≤ 0.05, ***P* ≤ 0.01, two-tailed *t* test.

The Upstream and Downstream of the RPA–RNF20–SNF2H Cascade Diverges at Centromeres and DNA Breaks. Although the RPA–RNF20–SNF2H cascade is a common mechanism shared by chromosome segregation and HR repair, the upstream and downstream of this pathway diverge between the two processes. First, the localization of RPA to centromeres relies on the presence of R-loops, while RPA loading at DSBs depends on ssDNA generated by DNA end resection. Second, the RPA–RNF20–SNF2H cascade regulates chromosome segregation by controlling the centromeric localization of RSF1 and, thereafter, the activation of Aurora B kinase. However, its role in HR seems to depend on the chromatin relaxation activity of SNF2H rather than on RSF1 (41, 49). In contrast to SNF2H, RSF1 appears to act by facilitating the deposition of centromere proteins CENP-S and CENP-X at DSBs or recruiting HDAC1 to promote transcriptional repression at DSBs (78–80). Thus, SNF2H and RSF1 exert different functions in DNA repair (40, 41, 43, 49, 79).

Roles of RNF20 in Tumorigenesis and Cancer Therapy. As a tumor suppressor, RNF20 is associated with cancer development, progression, and metastasis (81). Deregulation of RNF20 and H2Bub has been linked to multiple cancers, including lung, colorectal, and breast cancers (44, 81). Although RNF20 can regulate transcription, mRNA splicing, and DNA repair (44, 81), a full picture of its functions in suppressing genome instability and tumorigenesis is incomplete. Here, we reveal a unique function of the RPA–RNF20–SNF2H

cascade in regulating kinetochore–microtubule attachments and coupling H2Bub to chromosome segregation and DNA repair. Disruption of the RPA–RNF20 interaction results in chromosome missegregation, HR deficiency, and genome instability, which are hallmarks and vulnerabilities of cancer cells. The dual roles of the RPA–RNF20–SNF2 cascade in counteracting DNA damage and chromosome instability make the pathway an attractive therapeutic target in cancer treatment. However, we cannot exclude the possibility that RNF20 may also target other substrates to promote DNA repair or chromosome segregation.

Materials and Methods

Cell Culture and Drug Treatments. HEK293T, HeLa, HCT116, and U2OS cells were grown in Dulbecco's modification of Eagle's medium (DMEM) or McCoy's 5A with 10% FBS and 1% penicillin/streptomycin at 37 °C and 5% CO_2. The U2OS DR-GFP and HeLa-H2B-GFP cell lines were kindly provided by Dr. Xingzhi Xu (Shenzhen University) and Dr. Fangwei Wang (Zhejiang University), respectively. The cells were synchronized by double-thymidine block to obtain an enriched population of M phase cells or in prometaphase with 0.33 μM nocodazole (Sigma). Mitotic cells were further enriched by "shake-off." Drugs used in this study, including HU, CPT, VP16, monastrol, IAA, and olaparib, were all obtained from Selleck or MedChemExpress.

Data, Materials, and Software Availability. All study data are included in the article and/or *SI Appendix*.

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