Histone chaperone FACT regulates homologous recombination by chromatin remodeling through interaction with RNF20

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RESEARCH ARTICLE

ABSTRACT

The E3 ubiquitin ligase RNF20 regulates chromatin structure through ubiquitylation of histone H2B, so that early homologous recombination repair (HRR) proteins can access the DNA in eukaryotes during repair. However, it remains unresolved how RNF20 itself approaches the DNA in the presence of chromatin structure. Here, we identified the histone chaperone FACT as a key protein in the early steps of HRR. Depletion of SUPT16H, a component of FACT, caused pronounced defects in accumulations of repair proteins and, consequently, decreased HRR activity. This led to enhanced sensitivity to ionizing radiation (IR) and mitomycin-C in a fashion similar to RNF20-deficient cells, indicating that SUPT16H is essential for RNF20-mediated pathway. Indeed, SUPT16H directly bound to RNF20 in vivo, and mutation at the RING-finger domain in RNF20 abolished its interaction and accumulation, as well as that of RAD51 and BRCA1, at sites of DNA double-strand breaks (DSBs), whereas the localization of SUPT16H remained intact. Interestingly, PAF1, which has been implicated in transcription as a mediator of FACT and RNF20 association, was dispensable for DNA-damage-induced interaction of RNF20 with SUPT16H. Furthermore, depletion of SUPT16H caused pronounced defects in RNF20-mediated H2B ubiquitylation and thereby, impaired accumulation of the chromatin remodeling factor SNF2h. Consistent with this observation, the defective phenotypes of SUPT16H were effectively counteracted by enforced nucleosome relaxation. Taken together, our results indicate a primary role of FACT in RNF20 recruitment and the resulting chromatin remodeling for initiation of HRR.

KEY WORDS: Chromatin remodeling, Homologous recombination, H2B ubiquitylation, RNF20, SUPT16H

INTRODUCTION

DNA double-strand breaks (DSBs) are the most deleterious form of DNA damage, and they can be caused by a plethora of sources, such as exposure to ionizing radiation (IR), radiomimetic compounds, or oxidative stress (Ward, 1988; Limoli et al., 2002). The DNA damage response (DDR), however, exists in the cell in order to circumvent such detrimental injuries (Ciccia and Elledge, 2010; Murr, 2010), in which the chromatin remodeling and DNA accessibility are the key early processes (Suganuma and Workman, 2011; Hargreaves and Crabtree, 2011). Such dynamics changes are often led by post-translational modifications (PTMs) (Zhou et al., 2011), including phosphorylation and ubiquitylation of the histone variant H2AX (Morrison et al., 2004), which occurs within minutes following exposure to IR and functions to recruit subsequent components, such as 53BP1, to sites of DSBs (Mallette et al., 2012). Previously, we and other groups have shown that the ubiquitin E3 ligase RNF20 accumulates at sites of DSBs and induces H2B ubiquitylation (Nakamura et al., 2011; Moyal et al., 2011; Chernikova et al., 2010). RNF20 is an ortholog of the budding yeast protein Bre1, and monoubiquitylates the histone H2B on Lys123 at regions of transcription elongation (Kao et al., 2004). In the DDR, this histone PTM has been shown to result in recruitment of an ATP-dependent chromatin remodeling factor, SNF2h (also known as SMARCA5), to sites of DSBs, followed by accumulation of BRCA1 and RAD51 (Nakamura et al., 2011). It has also been shown that RNF20 functions independently of the canonical H2AX phosphorylation (γH2AX) and the resulting 53BP1 accumulation, indicating that there is an alternative H2AX-independent pathway that operates in chromatin remodeling during the rejoining of DSBs. However, the mechanism that is responsible for the recruitment of RNF20 to the DNA damage site is still elusive.

Histone chaperones are known to loosen high-order nucleosome architecture and reorganize individual nucleosomes to provide an accessible DNA template for cellular machinery (Winkler and Luger, 2011). One of the histone chaperones, FACT (facilitates chromatin transcription), a heterodimeric complex comprising SUPT16H (the human ortholog of yeast Spt16) and SSRP1, has been implicated in nucleosome reorganization (Winkler and Luger, 2011; Orphanides et al., 1998). In transcription, FACT has been proposed to displace one H2A–H2B dimer from the nucleosome to allow for the passage of the transcribing RNA polymerase II through the nucleosomal template (Heo et al., 2008; Belotserkovskaya et al., 2003). However, recent studies support the model that FACT enhances ‘nucleosome breathing’ and stabilizes the open configuration of nucleosomes (Winkler and Luger, 2011; Formosa, 2012; Hondele et al., 2013). In this process, the transcription elongation requires ubiquitylation of H2B by RNF20–RNF40, which is engaged by the transcription elongation regulator PAF1 (Pavri et al., 2006; Kim and Roeder, 2009). PAF1 physically interacts with FACT in active genes (Ng et al., 2003; Piro et al., 2012), and recruits RNF20 to the promoter region, thus mediating indirect association of FACT with RNF20 (Orphanides et al., 2012).
et al., 1998). As a result, in transcription, RNF20 recruitment and the resulting H2B ubiquitylation are defective in cells lacking functional PAF1 (Reinberg and Sims, 2006).

In DNA repair, it has also been shown that H2B ubiquitylation is driven by RNF20–RNF40 to promote chromatin remodeling and the accumulation of homologous recombination repair (HRR) proteins at the sites of DSBs (Nakamura et al., 2011; Moyal et al., 2011). Similarly, yeast experiments have shown that the nucleosome displacement is a prerequisite for recruitment of HRR proteins, such as RAD51, to the sites of DSBs, as observed by histone H2B changes at the MATα locus (Tsukuda et al., 2005). In that regard, we herein investigated the role of FACT in the accumulation of RNF20 and HRR proteins during DNA repair. Our results show that upon generation of DSBs, SUPT16H interacted with RNF20, which was responsible for RNF20 localization to the chromatin and subsequent HRR. Intriguingly, such interaction between RNF20 and FACT did not depend on PAF1, suggesting a distinct mechanism from that observed in transcription.

RESULTS
SUPT16H is required for the recruitment of RNF20 in response to DNA damage
To investigate the involvement of SUPT16H in response to DNA damage, we first immunostained cells using antibodies against SUPT16H and γH2AX, a well-known marker for DSBs, following IR exposure. We found that SUPT16H, which initially localizes to the nucleoli, consistently formed foci, which partially colocalized with γH2AX (Fig. 1A). This limited colocalization could be due to the different kinetics of focus formation. Furthermore, we induced spatially localized DSBs in the cell by using focused micro-irradiation, which allowed us to observe a distinct portion of endogenous SUPT16H at the induced DNA damage tracks (supplementary material Fig. S1A), confirming that SUPT16H accumulates at the sites of DSBs. However, a time-lapse immunostaining analysis revealed that γH2AX foci peaked shortly after IR exposure, decreasing within the first hour, whereas SUPT16H focus formation occurred at a later time, but for a prolonged period (supplementary material Fig. S1B). Thus, it is possible that this difference in their recruitment kinetics might account for the limited colocalization observed in the results described earlier. Interestingly, the accumulation of SUPT16H at the damage sites was accompanied by a similar level of RNF20 accumulation (Fig. 1B; supplementary material Fig. S1C). To test whether SUPT16H function would rely on RNF20, we examined SUPT16H levels upon IR exposure in cells transfected with small interfering RNA (siRNA) against RNF20 (siRNF20). As shown in Fig. 1C (left panel, lanes 3 and 4), the presence of SUPT16H in the chromatin fraction was observed in both control and RNF20-depleted cells. However, when we transfected cells with short hairpin RNA (shRNA) against SUPT16H (shSUPT16H), RNF20 levels in the chromatin fraction were dramatically decreased, whereas no significant changes were observed in whole-cell extracts (Fig. 1C, right panel, lanes 7 and 8; supplementary material Fig. S1D, right panel, lanes 7 and 8). Our immunofluorescence experiments showed a similar outcome (supplementary material Fig. S1E). Interestingly, SUPT16H- or RNF20-depleted cells were equally sensitive to IR, and concurrent depletion of both factors did not cause any further sensitization (Fig. 1D).

We next reasoned that SUPT16H would affect events downstream of RNF20. Histone H2B ubiquitylation on lysine 120 (H2BK120), which is the ubiquitylation target of RNF20, was also decreased after RNF20 downregulation with either a mixture of three different sets of siRNA or each of them separately (supplementary material Fig. S1F,G). Similarly, a reduction of histone H3 dimethylation on lysine 4 (H3K4) was also observed. Moreover, to determine whether the H2B ubiquitylation and the subsequent H3K4 dimethylation were taking place at the DSBs, we analyzed their accumulation at the damage sites by a chromatin immunoprecipitation assay (ChIP) with an inducible DSB system (Niida et al., 2010). A DR-GFP reporter cassette was introduced into HeLa cells, and the DSBs were generated by transfection with the I-SceI endonuclease. Analysis revealed that abrogation of SUPT16H led to a reduction in the H2BK120 ubiquitylation and H3K4 dimethylation at the region next to the DSB sites, whereas in positions further away from the sites those differences were less pronounced than those in the control (Fig. 1E; supplementary material Fig. S1H). Taken together, these observations indicate that SUPT16H is crucial for RNF20 accumulation, and the subsequent histone modification at the DNA damage sites.

Owing to the fact that SUPT16H-depleted cells were sensitive to DNA-damaging agents, we speculated whether the absence of SUPT16H would compromise the recruitment of HRR proteins, such as RAD51 and BRCA1, to the damage sites. Transient knockdown of either SUPT16H or RNF20 led to an impairment in the formation of IR-induced RAD51 and BRCA1 foci when compared to their respective control (supplementary material Fig. S1J, K), whereas no cell disturbance was observed (supplementary material Fig. S1L). For further confirmation, we performed immunofluorescence to detect RNF20 and either BRCA1 or RAD51, and observed that cells lacking RNF20 also did not show formation of both IR-induced RAD51 and BRCA1 foci (supplementary material Fig. S1K). It has been reported that RAD51 and BRCA1 dysfunction significantly reduces HRR activity (Moyahan et al., 1999). For a more specific assessment of HRR activity, we used an Sc neo-containing reporter construct (Johnson et al., 1999), and as shown in Fig. 1F, HRR activity in SUPT16H-, as well as in RNF20-depleted cells, was significantly reduced. Importantly, concurrent depletion of both proteins did not lead to any further impairment in HRR activity, indicating that both proteins are components of a common pathway in the rejoining of DSBs through HRR.

SUPT16H interacts with RNF20 in DDR, independently from PAF1 and γH2AX
Although the FACT complex associates with RNF20 in transcription, their interaction is indirect and intermediated by PAF1 (Pavri et al., 2006; Kim and Roeder, 2009). Similarly, our results showed that under unstressed conditions, PAF1 did associate with SUPT16H (Fig. 2A, lanes 3 and 4). However, their interaction was dramatically reduced after treatment with actinomycin D (ActD), a transcription inhibitor, even in the presence of DNA damage (Fig. 2A, lanes 5 and 6). By contrast, co-immunoprecipitation analysis showed that there was an association between SUPT16H and RNF20 in the chromatin following the generation of DSBs. Surprisingly, although increasing concentrations of ActD (0.03 mg/ml and 0.2 mg/ml) did affect the overall interaction between SUPT16H and RNF20, their association following IR exposure was still observed (Fig. 2B, lanes 6 and 8). This is consistent with previous evidence showing that IR-induced H2B ubiquitylation was intact in the presence of different transcription inhibitors (Nakamura et al., 2011; Moyal et al., 2011). Similar results were observed.
Upon immunoprecipitation of anti-SUPT16H and blotting against RNF20 (supplementary material Fig. S2A). Thus, it is possible that some decline in SUPT16H and RNF20 interaction might be accounted for by the disturbance of DNA transcription, although DNA damage still induces their interaction.

To examine whether PAF1 would be responsible for the interaction of SUPT16H and RNF20 in DNA repair, we next transfected cells with siRNA against PAF1 (siPAF1) (supplementary material Fig. S2B) and performed immunoprecipitation experiments. Intriguingly, depletion of PAF1 did not abrogate the IR-induced interaction between SUPT16H and RNF20 (Fig. 2C), or their mobilization to the DSBs (Fig. 2D), although the overall interaction was slightly decreased, which is probably because of the indirect interaction of FACT and RNF20 via PAF1 (Piro et al., 2012). In support of this idea, H3K4 methylation, a marker for active transcription, was drastically decreased. 

Fig. 1. SUPT16H-dependent recruitment of RNF20 in response to DNA damage. (A) Immunostaining using anti-SUPT16H antibody revealed that SUPT16H formed discrete foci in U2OS cells 2 hours after exposure to 5 Gy, which partially colocalized with γH2AX. Scale bar: 10 μm. (B) SUPT16H colocalized with RNF20 following DNA damage. U2OS cells transfected with Flag-tagged RNF20 were irradiated with 5 Gy, and 2 hours later, immunostained using anti-SUPT16H and anti-Flag antibodies. Scale bar: 10 μm. (C) Accumulation of RNF20 at the chromatin was impaired in SUPT16H-depleted cells, whereas accumulation of SUPT16H remained unchanged in RNF20-depleted cells. HeLa cells were transfected with either shSUPT16H or siRNF20, and 2 hours after irradiation with 5 Gy, their chromatin fraction was assayed with immunoblotting analysis. γ-tubulin was used as a loading control. (D) Depletion of SUPT16H enhanced the sensitivity of MCF7 cells to IR, as determined by a colony assay 10 days following IR exposure. Results are means ± s.e.m. of three independent experiments. **P < 0.01.
reduced in siPAF1-transfected cells (supplementary material Fig. S2C). Further analysis using the DR-GFP reporter assay revealed that siPAF1-transfected cells did not display a significant perturbation in the efficiency of HRR activity, in contrast to SUPT16H or RNF20 depletion, or their concomitant knockdown (Fig. 2E). Also, no further significant sensitization to IR was observed in siPAF1-transfected cells, although depletion did induce some minor changes of S/G2, in which homologous recombination takes place (Fig. 2F, supplementary material Fig. S1K). These observations suggest that the mechanism of interaction between FACT and RNF20 might be a different one from that triggered during transcription, where PAF1 is essential for the indirect association of these two factors. 

We next sought to elucidate the functional relationship of SUPT16H in the rejoining of DSBs through modifications of H2AX. To that end, we depleted SUPT16H and observed that it did not affect IR-induced γH2AX (supplementary material Fig. S2D,E). Moreover, H2AX-dependent recruitment of 53BP1 formed the expected number of foci in both SUPT16H- and RNF20-depleted cells (supplementary material Fig. S2E). Additionally, H2AX depletion by siRNA did not affect SUPT16H accumulation after laser micro-irradiation.
Intriguingly, RNF20 (Kim et al., 2009; Hwang et al., 2003). This evidence was further RING-finger domain of E3 ligases is generally believed to serve for the interaction between RNF20 and SUPT16H. Although the Mailand et al., 2007), showed that RING-finger domain is crucial for the interaction between RNF20 and the downstream HRR proteins in DNA repair. Given the function of FACT to sequester core histones from the relaxation agent, but not otherwise (Fig. 4A). Consistent with these observations, laser micro-irradiation analysis revealed that SNF2h accumulation at the damage sites was substantially reduced in RNF20-depleted cells (Fig. 4D). Importantly, cells lacking SUPT16H also failed to accumulate SNF2h. Conversely, in PAF1-depleted cells, SNF2h seemed to localize normally at the irradiated sites, suggesting that SUPT16H is crucial for SNF2h accumulation at damage sites, whereas PAF1 was again dispensable for such events. Taken together, these results indicate that FACT mediates RNF20 accumulation following DNA damage and, as a result, the chromatin remodeling that is important for the recruitment of the repair machinery to sites of DSBs in order to facilitate HRR.

**DISCUSSION**

FACT is a protein complex responsible for nucleosome reorganization during DNA transactions and further protein recruitment to the target site (Tan et al., 2006; Wittmeyer and Formosa, 1997; Kim et al., 2009), and it directly interacts with gene transcription factors, including RNA polymerase II and PAF1 (Pavri et al., 2006; Kim et al., 2009), and with DNA replication factors, such as DNA polymerase alpha and MCM helicase (Tan et al., 2006; Wittmeyer et al., 1997). We showed here that SUPT16H interacts with the RING-finger domain of RNF20 upon DNA damage, facilitating the chromatin remodeling for HRR independently of PAF1 and H2AX phosphorylation.

We found that SUPT16H formed foci following IR exposure. When SUPT16H was abrogated, cell survival following DNA stress drastically reduced, characterized by a reduction of HRR activity. Interestingly, SUPT16H foci were colocalized with those of RNF20 (Fig. 1B), and concomitant depletion of these two proteins did not cause any further sensitization under DNA stress (Fig. 1D) nor HRR activity in the cells (Fig. 1F). Taken together, these results suggest the participation of SUPT16H and RNF20 in the same HRR pathway. Furthermore, there seems to be an upstream function for FACT, because IR-induced focus formation of RNF20 was impaired in the absence of SUPT16H, but not otherwise (supplementary material Fig. S1E). This was further supported by co-immunoprecipitation experiments where we showed that SUPT16H physically interacted with RNF20 through its C-terminus RING-finger domain (Fig. 3C). Mutation in this domain compromised the formation of RNF20 foci, and also those of RAD51 and BRC1 foci were also defective in RNF20RING and RNF20RING, RNF20RING-transfected cells (Fig. 3E). These observations enforce the idea of interaction of SUPT16H and RNF20 at the RING-finger domain, which is essential for recruitment of RNF20 and the downstream HRR proteins in DNA repair.

**The SUPT16H-defective phenotype is counteracted by chromatin relaxation**

Given the function of FACT to sequester core histones from the nucleosome to allow for the passage of transcription machinery (Heo et al., 2008), we examined whether the impairment of DSB repair resulting from the lack of SUPT16H could be reversed by nucleosome relaxation. We observed that IR-induced RNF20 focus formation in SUPT16H-depleted cells seemed to recover when cells were pretreated with chloroquine, a nucleosome relaxation agent, but not otherwise (Fig. 4A). Consistent with this, accumulation of RNF20 and RAD51 at the chromatin fraction, and H2B ubiquitylation, were alleviated in SUPT16H-depleted cells pretreated with chloroquine (Fig. 4B, lanes 3, 4, 7 and 8; supplementary material Fig. S4A), as were RAD51 and BRC1 foci formation (Fig. 4C; supplementary material Fig. S4B). Similar results were observed in cells pretreated with a histone deacetylase inhibitor, trichostatin A (TSA), and hypotonic buffer, which also promote chromatin relaxation (Bakkenist and Kastan, 2003; Krajewski, 1999) (supplementary material Fig. S4C,D).

Recent reports have shown that deletion of SNF2h, a subunit of the ISWI chromatin remodeling complex (Santos-Rosa et al., 2003), confers cells with high sensitivity to DNA-damaging agents (Yoshimura et al., 2009; Smeenk et al., 2012; Nakanishi et al., 2007). We have previously shown that SNF2h is regulated by RNF20-dependent H2B ubiquitylation (Nakamura et al., 2011). Consistent with these observations, laser micro-irradiation analysis revealed that SNF2h accumulation at the damage sites was substantially reduced in RNF20-depleted cells (Fig. 4D). Importantly, cells lacking SUPT16H also failed to accumulate SNF2h. Conversely, in PAF1-depleted cells, SNF2h seemed to localize normally at the irradiated sites, suggesting that SUPT16H is crucial for SNF2h accumulation at damage sites, whereas PAF1 was again dispensable for such events. Taken together, these results indicate that FACT mediates RNF20 accumulation following DNA damage and, as a result, the chromatin remodeling that is important for the recruitment of the repair machinery to sites of DSBs in order to facilitate HRR.
Fig. 3. SUPT16H physically interacts with the RNF20 RING-finger domain. (A) Schematic of the domain architecture of RNF20 and SUPT16H and their respective mutants as used in this study. N, SUPT16H N-terminus; MID, SUPT16H mid-terminus; C, SUPT16H C-terminus. (B) SUPT16H interacted with RNF20 through its C-terminus. Analysis was performed using HA–RNF20 and Flag–SUPT16H recombinant sequences. Flag M2 beads were used to immunoprecipitate Flag-constructs harboring SUPT16HΔN, SUPT16HΔMID or SUPT16HΔC mutants and further blotted against anti-HA antibody for RNF20 detection. (C) RNF20 interacted with SUPT16H through its RING-finger domain. The chromatin fractions of Flag-tagged RNF20ΔC, RNF20ΔN, RNF20Δ1, RNF20Δ2, RNF20Δ3, RNF20ΔRING and RNF20C922S cells were co-immunoprecipitated with Flag M2 beads. (D) IR-induced RNF20 foci were compromised in both RNF20ΔRING and RNF20C922S-transfected cells, although IR-induced SUPT16H foci remained unchanged. U2OS cells expressing siRNA-resistant Flag-tagged RNF20 mutants were transfected with siRNF20, prior to exposure to 5 Gy, and 2 hours after irradiation, immunostained with anti-Flag and anti-SUPT16H antibodies. Scale bar: 10 µm. (E) The RING-finger domain of RNF20 is crucial for proper formation of IR-induced foci containing RAD51 and BRCA1. U2OS cells expressing siRNA-resistant Flag-tagged RNF20 mutants were transfected with siRNF20, prior to exposure to 5 Gy, and 2 hours after irradiation, immunostained with anti-RAD51 and anti-BRCA1 antibodies. (-) represents transfection with empty vector. Scale bar: 10 µm.
that the knockdown of RNF40 affects SUPT16H accumulation after treatment with radiomimetics, possibly because of an indirect effect of the decreased transcription activity (Kari et al., 2011). However, we demonstrated here that FACT binds to RNF20 upon formation of DSBs through its C-terminal domain (Fig. 3B), which encompasses most of its functional domains (Hondele et al., 2013; Myers et al., 2011). Surprisingly, such interaction was not dependent on transcription, nor mediated by PAF1 (Fig. 2). This is further supported by previous studies showing that RNF20-dependent H2B ubiquitylation is intact in the presence of different transcription inhibitors (Nakamura et al., 2011; Moyal et al., 2011). Importantly, such results do not contradict those observed previously (Fink et al., 2007), once the overall level of interaction between SUPT16H and RNF20, and the resulting H3 dimethylation on lysine 4 were slightly reduced by both PAF1 depletion and transcription inhibition (Fig. 2B,C; supplementary material Fig. S2C). Moreover, abrogation of PAF1 did not affect the radiation sensitivity of the cells (Fig. 2F), in accordance with previous observations (Game et al., 2006). These findings suggest that the mechanism of interaction between

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**Fig. 4. SUPT16H-depleted phenotype was recovered by chromatin relaxation.** (A) Chloroquine rescued the localization of RNF20 in SUPT16H-depleted cells. U2OS cells were treated with chloroquine (20 μg/ml), prior to exposure to 5 Gy of IR, and 2 hours later, immunostained with anti-RNF20 and anti-SUPT16H antibodies. Scale bar: 10 μm. (B) Chloroquine treatment significantly rescued the impaired accumulation of RNF20 and ubiquitylation of H2B (H2Bub) in the chromatin fraction. HeLa cells transfected with shSUPT16H were treated with chloroquine, prior to exposure to 5 Gy, and 2 hours later, their nuclear fraction was analyzed by western blotting. γ-tubulin was used as a loading control. (C) Chloroquine treatment leads to a significant recovery in the impaired formation of IR-induced RAD51 foci in SUPT16H-depleted cells. U2OS cells transfected with siRNF20 or shSUPT16H were treated with chloroquine (20 μg/ml), prior to exposure to 5 Gy of IR, and immunostained with anti-RAD51 antibody. Foci-positive cells were counted and results are means ± s.e.m. of three independent experiments. *P<0.05; **P<0.01. (D) Accumulation of SNF2h at laser-induced damage sites was impaired in FACT- and RNF20-depleted cells, whereas no significant difference was observed in siPAF1-transfected cells. U2OS cells were fixed 30 minutes after laser irradiation, and stained with the anti-GFP and anti-γH2AX antibodies. γH2AX was used as a DNA damage readout. Scale bar: 10 μm.
FACT and RNF20 is a different one from that triggered during transcription, where PAF1 is essential for the indirect association of these two factors. Although H2B is also known to interact with both proteins, it is unlikely that it exclusively mediates their interaction, at least in transcription. Previous studies have shown that only when PAF, a possible mediator of the interaction between SUPT16H and RNF20, was added to their reconstituted transcription assay, did they observe a robust transcription level, showing that the presence of H2B by itself was not sufficient for it (Pavri et al., 2006). Nonetheless, further investigation is needed to help elucidate how this mechanism takes place in DNA repair.

In DNA repair, the canonical signaling pathway is initiated by the phosphorylation of H2AX, leading to the accumulation of the subsequent repair-related proteins. The present results indicate that H2AX phosphorylation, and the resulting accumulation of 53BP1 at the chromatin remained unchanged after depletion of FACT (supplementary material Fig. S2D,E), and that, similarly, depletion of H2AX did not affect SUPT16H accumulation after generation of DNA damage (supplementary material Fig. S2F). These observations are consistent with evidence indicating that FACT is in the same pathway with RNF20, which is independent from H2AX phosphorylation, in DNA repair (Nakamura et al., 2011; Moyal et al., 2011). In accordance with this, another study has shown that inhibition of H2A phosphorylation in yeast, by a replacement of H2A by itself, was not sufficient for H2A to help elucidate how this mechanism takes place in DNA repair.

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Recently, a global accessibility/non-eviction model has been proposed for FACT, which suggests that FACT stabilizes the open configuration of nucleosomes and, hence, H2A–H2B dimer displacement could be a nonessential byproduct of FACT action (Winkler and Lugner, 2011; Formosa, 2012). This model is supported by a genetic study, which showed that the Spt16-defective phenotype was suppressed by a histone mutation that supported by a genetic study, which showed that the Spt16-defective phenotype was suppressed by a histone mutation that promoted increased levels of the open-reorganized form of nucleosomes (McCullough et al., 2011). In a similar manner, our results after treatment with different types of chromosome relaxing agents, chloroquine, TSA and hypotonic solution (Bakkenist and Krajewski, 1999), suppressed the SUPT16H-defective phenotype (Fig. 4, supplementary material Fig. S4). Thus, the open nucleosomal form might be able to recruit more FACT upon generation of DSBs, but independently from transcription. Taken together, these results suggest that FACT recruits RNF20 through its nucleosome reorganization activity and thereby facilitates chromatin remodeling, which is then mediated by RNF20 and SNF2h (Fig. 4D).

Consequently, the present observations demonstrate a role for SUPT16H outside its well-established function in transcription. SUPT16H initiates HRR through RNF20-dependent H2B ubiquitination, and its depletion compromises HRR activity. In so much as the defect in HRR is able to trigger sufficient genomic instability to accelerate cancer-promoting mutations (Cousineau and Belmaaza, 2007), the dysfunctional SUPT16H pathway could conceivably increase cancer susceptibility. Consistent with this prediction, recent studies have shown that tumorigenesis is promoted by dysfunction of Smurf2, a HECT-domain E3 ubiquitin ligase, which induces RNF20 degradation by proteasomal machinery (Blank et al., 2012). Similarly, CDC73, which regulates H2B ubiquitylation through its interaction with RNF20, is mutated and/or downregulated in various types of tumors (Hahn et al., 2012). Therefore, our study underscores the importance of chromatin remodeling during DNA repair, and thus maintenance of genomic stability, driven by a PAF1-independent interaction between FACT and RNF20.

**MATERIALS AND METHODS**

**Cell cultures, RNAi constructs and transfection**

Human HeLa, U2OS, MCF7 and 293E cells were grown in DMEM with 10% fetal bovine serum supplemented with antibiotics and incubated at 37°C. A mixture of RNA oligonucleotides (for inhibition of RNF20 no. 1, 5'-GCUAAAAGAGUCAGAAATTG-3'; no. 2, 5'-UGGAAGAGAU-AAGGAAUATTG-3'; no. 3, 5'-GAAGAAUCUUGGGUAUTT-3'; 3 μg/ml), for inhibition of PAF1(no. 1, 5'-UGGAAGACUUUGA-GGAAUATT-3'; no. 2, 5'-GGGACCCAGGAGGAGAGAUUTT-3'; no. 3, 5'-GCUCAGAGUCUUGCUUGTT-3', 3 μg/ml for inhibition of H2AX), (5'-CACAAGGAAGAGCGAAUUTT-3'; B-Bridge, Cupertino, CA), a short-hairpin RNA placid (1 μg/ml) for inhibition of SUPT16H (5'- GGAAGAATGATGAGGAGGA-3'), and non-targeting short-interfering RNAs (B-Bridge) were transfected into cells using Lipofectamine 2000 (Invitrogen, San Diego, CA), according to the manufacturer’s protocol. At 2 days after siRNA/shRNA transfection, transfected cells were used for following experiments.

**Plasmid and DNA manipulation**

Wild-type and resistant full-length RNF20 and its respective mutant cDNAs were N-terminally tagged with either FLAG or HA epitopes, and subcloned into a pcDNA3 plasmid backbone (Invitrogen). SUPT16H N-, MID- and C-termini cDNAs were N-terminally tagged with FLAG epitope and subcloned into a pcDNA3 plasmid backbone (Invitrogen). Mutant RNF20 and SUPT16H constructs were generated by PCR, employing Pyrobest DNA polymerase (Takara Bio Inc., Otsu, Japan), using primers containing the respective mutations within the sequence. The insert DNA was then confirmed by DNA sequencing.

**SCneo and DR-GFP assays**

The SCneo assay was used for site-specific DNA DSB repair analysis, performed in HeLa-SCneo cells as described previously (Johnson et al., 1999). Briefly, the SCneo plasmid was transfected into cells by electroporation, and stable transformants, with one copy of the SCneo, were isolated. For induction of DSBs at the I-Sce I site, pCßASce plasmid was electroporated for induction of DSBs at the I-Sce I site, and cells were then incubated for 48 hours and subsequently grown in medium containing 1.6 mg/ml of G418 (Calbiochem). Similarly, stably transfected HeLa cells with the DR-GFP HRR reporter cassette were assessed as described previously (Pierce et al., 1999). The DR-GFP plasmid was transfected into the cells by electroporation, and stable transformants, with one copy of the SCneo, were isolated. For induction of DSBs at the I-Sce I site, pCßASce plasmid was electroporated for induction of DSBs at the I-Sce I site, and cells were transfected for 48 hours. GFP-positive cells were assessed using a FACSAria flow cytometer equipped with CellQuest software (Becton Dickinson).

**Treatment with inhibitors and chromatin-modifying agents**

HeLa cells were treated with actinomycin D (30 μg/ml) 1 hour before irradiation, and harvested 2 hours later for western blotting. Cells were treated with trichostatin A (TSA) (0.2 mM) and hypotonic solution (50 mM salt solution in PBS pH 7.4), for 6 hours, and chloroquine (20 mg/ml), for 2 hours, and harvested 2 hours following exposure to IR for further western blotting and immunostaining analyses.

**Preparation of chromatin fraction**

Cells were treated with actinomycin D (30 μg/ml or 200 μg/ml, where specified) 2 hours before irradiation, and then harvested for further
analysis. Chromatin fractions were isolated according to the method of Méndez and Stillman (Méndez and Stillman, 2000). Briefly, cells were lysed in hypotonic buffer [10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.34 M sucrose, 10% glycerol, complete protease inhibitor cocktail (Roche), 1 mM DTT and 0.1% Triton X-100] to isolate nuclei. Nuclei were then resuspended in extraction buffer [3 mM EDTA, 0.2 mM EGTA, complete protease inhibitor cocktail (Roche), 1 mM DTT]. Insoluble chromatin was collected by centrifugation and sheared by sonication.

ChIP assay
To generate DSBs by means of the I-SceI restriction enzyme, 1 × 10⁶ stably transfected HeLa-DRGF cells were pre-treated with 10 µM of Nu7026 (DNA-Pkcs inhibitor; Calbiochem) for 1 hour and then, 50 µg of the I-SceI expression vector pC6ASce was introduced to them by electroporation (BIO-RAD). 24 hours later cells were treated with 1% formaldehyde at 37°C for 10 minutes, followed by the addition of 0.125 M glycine. The ChIP assay was performed essentially as described previously (Nakamura et al., 2011). Cells were used for immunostaining, immunoprecipitation and western blotting analysis.

Immunoprecipitation
For the immunoprecipitation assay, cells were washed with cold PBS and lysed on ice for 30 minutes in IP-buffer [20 mM HEPES-NaOH (pH 7.4), 0.2% NP-40, 150 mM NaCl, 25% glycerol, 1 mM EDTA, protease inhibitor, 10 mM NaF, 1 mM Na₂VO₃]. Soluble material was recovered by centrifugation at 3000 g for 5 minutes. The pellet was washed twice with lysis buffer and extracted with IP buffer for 30 minutes on ice. After sonication and subsequent centrifugation, this chromatin fraction was diluted with IP buffer and proteins were immunoprecipitated for 2 hours with the respective antibodies. The precipitates were washed and resuspended in loading buffer, boiled, and then submitted to western blotting analysis. In the case of the tagged-antibodies, the human GAPDH locus was amplified using whole genomic DNA with GAPDH-F (5'-CATGCCGAGGGCTA-CGT-3') and Sce180-R (5'-CGGCGGGCTTCTGC-3'), Sce600-F (5'-TCCGCCCTGAGCAAAGAC) and Sce600-R (5'-ACGAACCTCCAG-AGGACCAT-3'), Sce3100-F (5'-CCCCAGTCTAGTGTCCGCTTCTT-3') and Sce3100-R (5'-CTTCCGGACCTTTCTCTTCTC-3'). As an internal control for the normalization of the specific fragments amplified, the human GAPDH locus was amplified using whole genomic DNA with GAPDH-F (5'-TCTCCGCCACACATCGACCT-3') and GAPDH-R (5'-CCTAGTCCCGGCGTTTTATTG-3'). The efficiency of DSB induction was ~2–3%. The relative accumulations of proteins are defined as the ratio of accumulated protein amounts in the presence of DSB (cut by I-SceI) with that in the absence of DSB (uncut).

Microirradiation analysis
Cells stably expressing GFP-tagged NBS1 and SNF2h were treated with Hoescht 33258 (final concentration 4 µg/mL) for 10 minutes and were microirradiated with a 405-nm laser coupled into a Leica TCS SP5 confocal laser-scanning microscope, as described previously (Nakamura et al., 2011). Cells were used for immunostaining analysis.

Western blotting and immunostaining analysis
Western blotting was carried out as described previously (Nakamura et al., 2011) using the indicated antibodies. For immunostaining analysis, cells on coverslips were washed with PBS, fixed in 4% formalin and incubated with a detergent solution at 4°C for 5 minutes. After treatment with a blocking solution (3% low-fat milk) for 30 minutes, cells were

exposed to the appropriate primary antibody, and the Alexa-Fluor-488 and Alexa-Fluor-546-conjugated fluorescent secondary antibodies (Invitrogen). After washing, the cells were mounted on a slide glass. Fluorescence was visualized with a confocal laser-scanning microscope (Olympus FV300). At least 200 cells were counted for quantification of focus-positive cells.

Antibodies
The following antibodies were used for immunostaining, immunoprecipitation and western blotting analysis: polyclonal anti-SUP316H (Santa Cruz Biotechnology), anti-RNF20 (Bethyl), anti-H2AX (Abcam), anti-PAF1 (Bethyl), anti-H2B (Millipore), anti-H3K4me3 (Abcam), anti-RAD51 (Santa Cruz) and anti-HA (Sigma), and monoclonal anti-p-actin (Sigma), anti-γ-tubulin (Sigma), anti-BRCA1 (Calbiochem), anti-phospho-H2AX (Millipore), anti-H2BK120ab (Millipore), anti-H3 (Cell Signaling Technology), anti-Myc (9E10) (Covance) and anti-Flag M2 (Sigma).

Cell cycle analysis
Cells were fixed in ice-cold 70% ethanol. DNA was stained with 50 mg/ml propidium iodide (Sigma-Alrich) in PBS containing 0.1% Triton X-100 and 0.5 mg/ml DNase-free RNase A (Sigma-Aldrich). Samples were processed on a FACSCalibur flow cytometer equipped with CellQuest software (Becton Dickinson). The results were then analyzed using ModFit LT 3.1 software (Verity Software House).

Statistical analyses
The statistical significance of differences was determined using Student’s t-test. A P-value of <0.05 was considered to be significant.

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Competing interests
The authors declare no competing interests.

Author contribution
D.O. performed the experiments, analyzed the data and co-wrote the manuscript. A.K. contributed to the experiment with laser micro-irradiation. K.N., T.I. and H.T. contributed to the data analyses and discussion. M.O. contributed to the experiment with ChIP assay and colony assay, respectively. K.K. designed the study and co-wrote the manuscript.

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**Supplementary Figure 1.** SUPT16H functions in DNA damage response.

(A) Accumulation of a fraction of endogenous SUPT16H at laser-induced damage in U2OS cells 30 min after laser irradiation. The cells were stained with antibodies against SUPT16H (red) and the damage sensor NBS1 (green). Scale bar, 10 μm.

(B) Time-lapsed γH2AX and SUPT16H foci formation analysis. U2OS cells were irradiated with 5 Gy and harvested for immunofluorescence analysis at the respective time points. Error bars represent SEM of three independent experiments.

(C) SUPT16H- and RNF20-foci positive cells shown in Fig 1B were counted. Error bars represent SEM of three independent experiments.

(D) Whole cell extract from HeLa cells transfected with either shSUPT16H or siRNF20. Cells were submitted to 5 Gy and harvested 2 h later for immunoblotting analysis.

(E) SUPT16H-depletion compromised the formation of RNF20 foci, but not otherwise. U2OS cells expressing Flag-tagged RNF20 were transfected with either shSUPT16H or siRNF20, and 2 h after irradiation with 5 Gy, immunostained with anti-Flag and anti-SUPT16H antibodies. Scale bar, 10 μm.

(F) Depletion of SUPT16H inhibited the IR-induced ubiquitination of H2B and the subsequent H3K4 dimethylation. HeLa cells were transfected with shSUPT16H and 2 h after exposure to 5 Gy, their nuclear fraction was assayed with immunoblotting analysis. H3 was used as a loading control.

(G) Knockdown efficiency of the 3 different sets of siRNF20 oligonucleotides used as a mixture. HeLa cells were transfected with each set separately and assayed with immunoblotting analysis.

(H) ChiP analysis at distant positions (600 bp and 3100 bp, respectively) from I-Sce1-induced DSB sites. Soluble chromatin was immunoprecipitated by the anti-monoubiquitinated H2BK120 and anti-dimethylated H3K4 antibodies.

(I) IR-induced RAD51 and BRCA1 foci formations were impaired in RNF20- and SUPT16H-depleted cells. U2OS cells transfected with either siRNF20 or shSUPT16H were irradiated with 5 Gy, and 2 h later, immunostained using anti-RAD51 and anti-BRCA1 antibodies. Scale bar, 10 μm.

(J) RAD51- and BRCA1-foci positive cells shown in Fig. S1I were counted. Error bars represent SEM of three independent experiments. (**) p<0.01.

(K) The cell cycle distribution of SUPT16H-, RNF20- and PAF1-depleted MCF7 cells.

(L) IR-induced BRCA1 and RAD51 foci coincided with those cells with reminiscent RNF20 following transfection with siRNF20. U2OS cells expressing Flag-tagged RNF20 were transfected with siRNF20, and 2 h after irradiation with 5 Gy, immunostained with anti-Flag and either anti-BRCA1 or anti-RAD51 antibodies. Scale bar, 10 μm.
**Supplementary Figure 2.** SUPT16H DDR pathway does not depend on PAF1.

(A) Interaction of SUPT16H with RNF20 following DNA damage was not abrogated with ActD treatment. HeLa cells were treated with DMSO or ActD (30 μg/mL) prior to exposure to 5 Gy, and 2 h after irradiation, immunoprecipitation of the chromatin fraction with anti-SUPT16H was performed and blotted with anti-RNF20 antibody.

(B and C) HeLa cells were transfected with siPAF1, incubated for 72h, and irradiated with 5 Gy. Cells were harvest 2 h later and the protein fraction was immunoblotted against anti-PAF1, anti-SUPT16H and anti-RNF20 (B), and anti-H3K4diHMet antibodies (C).

(D and E) Normal phosphorylated ATM and 53BP1 foci formation 1h after 5Gy of IR treatment in SUPT16H- and RNF20-depleted U2OS cells, comparable to that observed in control siRNA-transfected cells.

(F) Accumulation of endogenous SUPT16H at laser-induced damage in siH2AX-transfected U2OS cells 30 min after laser irradiation. The cells were stained with antibodies against SUPT16H and the damage sensor NBS1. H2AX depletion was detected in SDS-PAGE gel. H3 was used as a loading control.

(G) SUPT16H knockdown leads to a prolonged presence of γH2AX foci following DNA damage. SUP16H-depleted U2OS cells were immunostained against γH2AX at the indicated time points following 5 Gy of ionizing radiation. Scale bar, 10 μm.
Supplementary Figure 3. IR-induced SUPT16H foci formation is not affected by disruption of RNF20’s RING-finger domain.

SUPT16H-foci positive cells shown in Figure 3D were counted. The analysis was confined to cells containing the respective transfected Flag-tagged RNF20 mutants. Error bars represent SEM of three independent experiments.
Supplementary Figure 4

(A) RAD51 accumulation and H2B ubiquitination were retrieved by chromatin relaxation. HeLa cells were treated with chloroquine (20 μg/mL), prior to exposure to 5 Gy of IR, and 2 h later, their nuclear fraction was analyzed with western blotting. H2B was used as a loading control. WCE represents the whole cell extract.

(B) Pretreatment with 20 μg/mL of chloroquine 4 h prior to exposure to 5 Gy alleviated the impairment of BRCA1 foci formation in shSUPT16H- or RNF20-transfected U2OS cells alone, or concomitant transfection. Error bars represent SEM of three independent experiments. (*) p<0.05

(C) Similarly, pretreatment with either 500nM of Trichostatin A, 6 h before exposure to 5 Gy, or Hypotonic solution, 4 h before irradiation, alleviated the impairment of both RAD51 (C) and BRCA1 (D) foci formation in shSUPT16H- and RNF20-transfected U2OS cells alone. Error bars represent SEM of three independent experiments. (*) p<0.05.