RNF168 forms a functional complex with RAD6 during the DNA damage response

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Summary

Protein ubiquitination plays an important role in initiating the DNA damage response. Following DNA damage, E2 ubiquitin conjugating enzymes are crucial for catalyzing substrate ubiquitination that recruits downstream DNA repair factors to DNA lesions. To identify novel E2 conjugating enzymes important for initiating the DNA-damage-induced ubiquitination cascade, we screened most of the known E2 enzymes and found that RAD6A and RAD6B function together with RNF168 in the ionizing radiation (IR)-induced DNA damage response. Similarly to RNF168-deficient cells, RAD6A- or RAD6B-deficient cells exhibit a reduction in DNA-damage-induced protein ubiquitination. Correspondingly, DNA-damage-induced foci formation of DNA damage repair proteins, such as BRCA1 and 53BP1, is impaired in the absence of RAD6A or RAD6B. Moreover, the RNF168–RAD6 complex targeted histone H1.2 for ubiquitination *in vitro* and regulated DNA-damage-induced histone H1.2 ubiquitination *in vivo*. Collectively, these data demonstrate that RNF168, in complex with RAD6A or RAD6B, is activated in the DNA-damage-induced protein ubiquitination cascade.

Key words: RAD6, RNF168, Ubiquitination, DNA damage response

Introduction

Cells encounter genotoxic stress on a regular basis, and are protected by the DNA damage response invoked after damage occurs. The most deleterious lesions caused by DNA damaging agents are DNA double strand breaks (DSBs). In response to DSBs, cells activate evolutionarily conserved cell cycle checkpoint pathways to arrest cell cycle progression and load DNA damage repair machinery at break sites in order to repair lesions (Rouse and Jackson, 2002; Sancar et al., 2004).

Recently, a unique protein ubiquitination cascade induced by DSBs was identified as an important signaling pathway that activates cell cycle checkpoints and DNA repair (Bennett and Harper, 2008; Bergink and Jentsch, 2009; Panier and Durocher, 2009). Several E3 ubiquitin ligases are sequentially activated to trigger these biological processes. Following DSBs, the RING domain E3 ligase RNF8 is recruited to DNA damage sites through the interaction between its FHA domain and phosphorylated MDC1, the partner of H2AX (Huen et al., 2007; Kolas et al., 2007; Mailand et al., 2007; Zhao et al., 2007). RNF8, in a complex with Ubc13, ubiquitinates histone H2A and initiates the ubiquitination cascade at DNA damage sites (Sakasai and Tibbetts, 2008; Wu et al., 2009; Zhao et al., 2007). The initial RNF8-dependent ubiquitination signals are then recognized by the ubiquitin-binding domain of RNF168, another RING domain E3 ligase (Doil et al., 2009; Stewart et al., 2009). RNF168 further induces protein ubiquitination events at DNA damage sites (Doil et al., 2009; Stewart et al., 2009). Moreover, HERC2, an E3 ligase partner of RNF8, and the downstream E3 ligase RAD18 also relocate to DNA damage sites and amplify the local ubiquitination events (Bekker-Jensen et al., 2010; Huang et al., 2009). These ubiquitination events are finally recognized by ubiquitin binding proteins, such as RAP80, which stabilizes other DNA damage response factors including BRCA1 and 53BP1 at DNA damage sites (Kim et al., 2007; Sobhian et al., 2007; Wang et al., 2007; Wu et al., 2009).

The function of RING domain E3 ligases is to link E2 ubiquitin conjugating enzymes to substrates. The RING domain E3 ligases do not have intrinsic enzymatic activity. It is the E2 conjugating enzymes that catalyze the ubiquitination of specific lysine residues on the substrate proteins. One well-studied E2 conjugating enzyme is Ubc13, the enzymatic partner of RNF8 (Campbell et al., 2012). Like RNF8, Ubc13 governs the DNA-damage-induced ubiquitination cascade, and is involved in DNA-damage-induced checkpoint activation and DNA damage repair (Huen et al., 2008; Wang and Elledge, 2007; Zhao et al., 2007). It has also been shown that Ubc13 is a partner of RNF168 during the DNA damage response, and was hypothesized to extend the ubiquitin chains initiated by the RNF8/Ubc13 complex (Doil et al., 2009; Stewart et al., 2009). However, recent structural and biochemical analyses indicate that RNF168 may function with other E2 partners in response to DNA damage (Campbell et al., 2012). To examine this possibility, we screened E2 enzymes and identified RAD6A and RAD6B as functional partners of RNF168 important for promoting the DNA damage response in response to DSBs.

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Results

To identify the possible E2 conjugating enzyme partners for RNF168, we cloned 30 non-redundant human E2 conjugating enzymes and examined their cellular localization (supplementary material Table S1). Following DNA damage, several E3 ligases are recruited to DNA damage sites via protein phosphorylation and ubiquitination events (Panier and Durocher, 2009). Following the relocation of E3 enzymes to DNA damage sites, the E2 enzymes are recruited to DNA damage sites and catalyze protein ubiquitination at DNA damage sites. However, the IRIF of endogenous E2 enzyme are relatively difficult to be observed because the interactions between E2 enzymes and E3 ligases are relatively transient and unstable *in vivo* (Deffenbaugh et al., 2003; Joazeiro and Weissman, 2000; Lorick et al., 1999; Pickart, 2001; Xie and Varshavsky, 1999; Ye and Rape, 2009). Thus, we overexpressed the E3 ligase to trap E2 enzymes at DNA damage sites, and examined if any of the E2 conjugating enzymes could form ionizing radiation (IR)-induced foci (IRIF) together with RNF168. Among these E2 conjugating enzymes, RAD6A and RAD6B formed IRIF when coexpressed with RNF168, suggesting that RNF168 could recruit RAD6A and 6B to DSBs (Fig. 1A). Interestingly, RAD6A and 6B failed to form clear IRIF with RNF8 (Fig. 1B). Although nuclear foci of RAD6A and 6B could be occasionally observed, these foci were not colocalized with RNF8. It is possible that spontaneous foci of RAD6A and 6B might be involved in other cellular processes. These results suggest that RAD6A and 6B could be novel E2 partners of RNF168 in response to DNA damage.

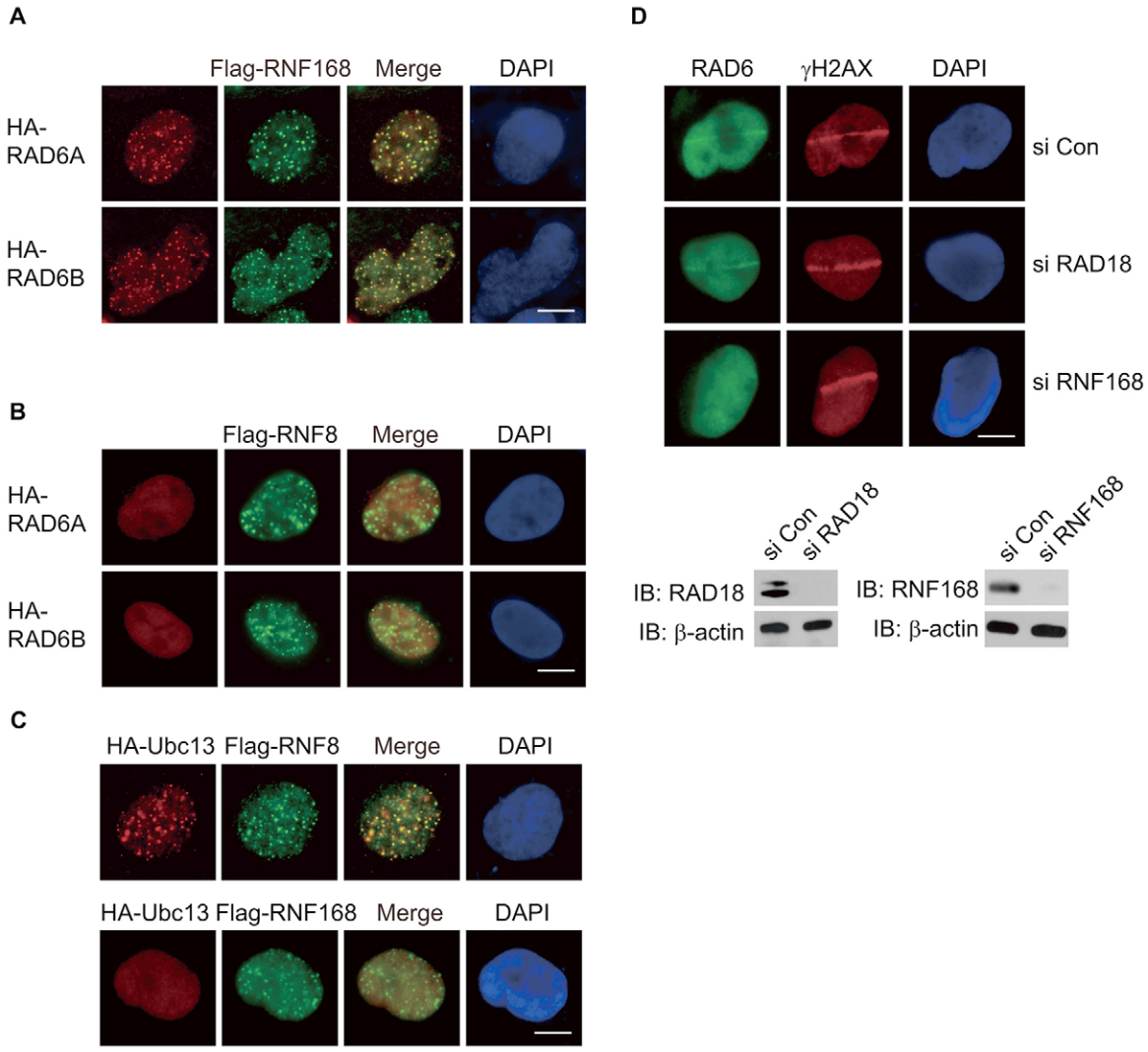

Fig. 1. RAD6A and RAD6B go to DNA damage sites together with RNF168. (**A**) RAD6A and 6B form IRIF together with RNF168. U2OS cells expressing HA-tagged RAD6A or 6B together with FLAG-tagged RNF168 were treated with IR (3 Gy). One hour later, cells were fixed and immunostained with polyclonal anti-HA and monoclonal anti-FLAG antibodies. (**B**) RAD6A and 6B do not form IRIF together with RNF8. U2OS cells expressing the indicated plasmids were treated with 3 Gy of IR and stained as for A. (**C**) Ubc13 forms IRIF when coexpressed with RNF8, but not with RNF168. U2OS cells expressing HA-tagged Ubc13 and FLAG-tagged RNF8 or RNF168 were treated with 3 Gy of IR and stained as for A. (**D**) U2OS cells were treated with the indicated siRNAs. After 48 hours, cells were subjected to laser microirradiation. After 30 minutes, cells were fixed and immunostained with anti-RAD6 and anti-γH2AX antibodies. The RNF168 and RAD18 protein expression after siRNA treatment were detected by western blot. si Con, control siRNA; si RNF168, RNF168 siRNA; si RAD18, RAD18 siRNA. Scale bars: 10 μm.

Based on the structural analysis of E2/E3 interactions (Zheng et al., 2000), the key residues for E2 conjugating enzyme interaction in the RING domains of RNF8 and RNF168 are quite different, providing an explanation for the specific binding of RAD6A and 6B to RNF168. While Ubc13 was recently identified as an E2 partner for both RNF8 and RNF168, this may be a unique occurrence. We examined the IRIF of Ubc13, and found that Ubc13 formed clear IRIF when Ubc13 coexpressed with RNF8 (Fig. 1C), indicating that RNF8 recruits Ubc13 to DNA damage sites, which is in agreement with previous studies (Huen et al., 2007; Kolas et al., 2007; Mailand et al., 2007; Wang and Elledge, 2007). Interestingly, we found that Ubc13 failed to form clear IRIF when coexpressed with RNF168 (Fig. 1C), leading us to the question if the RNF168/Ubc13 complex recruitment could be a secondary event following a primary RNF168-dependent protein ubiquitination at DNA damage sites, which is also supported by a recent structural analysis of the RING domain of RNF8 and RNF168 (Campbell et al., 2012).

Like RNF168, RAD6 relocated to DNA damage sites generated by laser microirradiation (Fig. 1D). Different from low dose IR, laser microirradiation induces huge amount of DNA damage within a very limited area. Thus, we could observe that endogenous RNF6 was recruited to DNA damage sites. It has been reported that RAD18, an enzymatic partner of RAD6 (Haracska et al., 2006; Hoege et al., 2002), is recruited to DNA damage sites and directly binds to ubiquitinated H2A after DNA damage (Inagaki et al., 2011; Watanabe et al., 2009). To exclude the possibility that RAD6 is recruited by RAD18 after DNA damage, we depleted RAD18 by siRNA treatment and found that RAD6 was still recruited to DNA damage sites (Fig. 1D and supplementary material Fig. S1). However, when RNF168 is depleted by siRNA treatment, the relocation of RAD6 to DNA damage sites was largely suppressed, suggesting that RNF168 controls the recruitment of RAD6 to DNA lesions (Fig. 1D).

The primary sequences of RAD6A and 6B are nearly identical, and they are derived from the same ancestor, RAD6, in yeast (Koken et al., 1991). To further examine whether RAD6A and 6B specifically interact with RNF168, we used recombinant RAD6A and 6B to pull down RNF168 and found that RAD6A and 6B interacted with RNF168 (Fig. 2A). In contrast, neither RAD6A nor 6B could pull down RNF8 from cell extracts (Fig. 2B). Based on the structural analysis of E2/E3 interactions, Ile18 in the RING domain of RNF168 is a key residue for the interaction with E2 conjugating enzymes, and an Ile-to-Ala mutation (I18A) disrupts the interaction while leaving the tertiary structure of the RING domain intact (Brzovic et al., 2001; Zheng et al., 2000). As shown in Fig. 2C, the I18A mutation abolished the interaction between RNF168 and RAD6A or 6B. Conversely, we mutated Pro64, Asn65 and Pro68 to Ala in RAD6A and 6B, the putative interaction sites with E3 ligases (Zheng et al., 2000). Mutant RAD6A and 6B failed to interact with RNF168 (Fig. 2D). To investigate whether RAD6 interact with RNF168 *in vivo*, we did co-immunoprecipitation (co-IP) assays. Since the interaction between E2 conjugase and E3 ligase is usually very transient (Deffenbaugh et al., 2003; Joazeiro and Weissman, 2000; Lorick et al., 1999; Pickart, 2001; Xie and Varshavsky, 1999; Ye and Rape, 2009), we treated cells with dithiobis(succinimidyl propionate) (DSP) to crosslink the unstable E2/E3 complex. RAD6 co-immunoprecipitated with RNF168, but not RNF8 following IR treatment (Fig. 2E). Although RAD18 was also associated with RAD6, it was not in the same complex with RNF168 (Fig. 2E). Next, we coexpressed HA-tagged RAD6A or

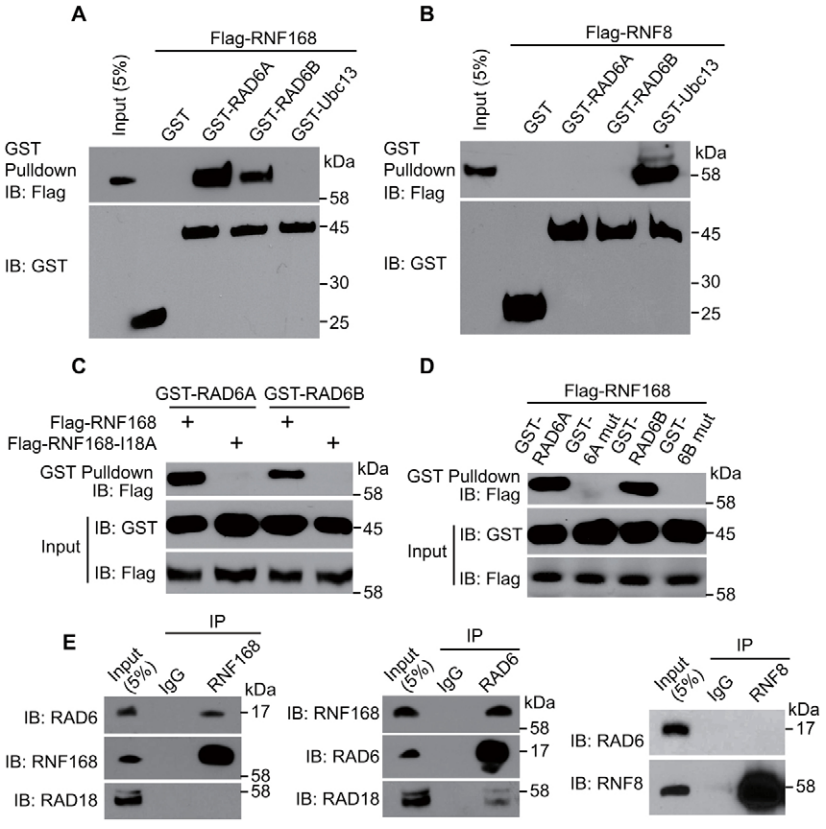

Fig. 2. RAD6A and RAD6B interact with RNF168 *in vitro* and *in vivo*. (**A**) RAD6A and 6B interact with RNF168. FLAG-tagged RNF168 were transiently expressed in 293T cells. After 24 hours, cell lysates were subjected to GST pulldown assay with the indicated GST fusion proteins. (**B**) RAD6A and 6B cannot interact with RNF8. FLAG-tagged RNF8 was transiently expressed in 293T cells. After 24 hours, cell lysates were subjected to GST pulldown assay with the indicated GST fusion proteins. (**C**) The RING domain of RNF168 interacts with RAD6A and 6B. FLAG-tagged wild-type or I18A mutant of RNF168 were transiently expressed in 293T cells. After 24 hours, cell lysates were subjected to GST pulldown assay with the indicated GST fusion proteins. (**D**) Key residues in RAD6A and 6B are important for the interaction with RNF168. FLAG-tagged RNF168 was expressed in 293T cells. After 24 hours, cell lysates were subjected to GST pulldown assay with the indicated GST fusion proteins. GST-6A mut and GST-6B mut refer to GST-RAD6A or GST-RAD6B with Pro64, Asn65 and Pro68 to Ala mutations. (**E**) RAD6 interacts with RNF168, not RNF8 *in vivo*. 293T cells were treated with 10 Gy of radiation. After 30 minutes, cells were crosslinked with DSP. Co-immunoprecipitation was then performed using control IgG, anti-RNF168, anti-RAD6 or anti-RNF8 antibodies. The associated proteins were then blotted with anti-RAD6, anti-RNF168, anti-RAD18, and anti-RNF8 antibodies.

RAD6B together with RNF168 I18A mutant, which cannot interact with RAD6. HA-RAD6A or RAD6B could not form IRIF. Consistently, RAD6A or RAD6B mutants that abolish the interaction with RNF168, failed to form IRIF either, suggesting that the interaction between RNF168 and RAD6A or RAD6B is important for recruiting RAD6A and RAD6B to DNA damage sites (supplementary material Fig. S2). Taken together, these results suggest that RAD6A and 6B interact with RNF168, but not RNF8, and this interaction occurs through the RING domain of RNF168 and the E3 ligase interaction sites identified by Zheng et al. on RAD6A and 6B (Zheng et al., 2000).

To study the role of RAD6 in the DNA-damage-induced ubiquitination cascade, we first examined IRIF of ubiquitin. As shown in Fig. 3A, IRIF of ubiquitin was modestly impaired in *Rad6A*$^{-/-}$ or *Rad6B*$^{-/-}$ cells. *Rad6A*$^{-/-}$ or *Rad6B*$^{-/-}$ cells reconstituted by wild-type RAD6A or RAD6B, but not the mutants (Pro64, Asn65 and Pro68 to Ala) that abolish the interaction with RNF168, could restore the IRIF of ubiquitin (Fig. 3B). Moreover, depletion of RAD6A or RAD6B by siRNA in U2OS cells also impaired the IRIF of ubiquitin with the reduction of both intensity and quantity of Ub foci. Knockdown of both RAD6A and 6B additively impaired the ubiquitin foci formation (Fig. 3C). Reconstitution of wild-type RAD6A or RAD6B, but not the mutants in these double depleted cells, could restore the IRIF of ubiquitin (supplementary material Fig. S3). Collectively, these results suggest that RAD6A and 6B are important in the DNA-damage-induced ubiquitination cascade. Next, we examined the IRIF of several DNA damage response factors in *Rad6A*$^{-/-}$ and *Rad6B*$^{-/-}$ cells. Following IR, γH2AX and MDC1 provide platforms to recruit and stabilize other DNA damage response factors such as RNF8 at DSBs (Huen et al., 2007; Mailand et al., 2007). It is RNF8 and the RNF8-dependent ubiquitination cascade that govern the IRIF of downstream DNA damage response mediators such as BRCA1 and 53BP1 (Huen et al., 2007; Kolas et al., 2007; Mailand et al., 2007; Wang and Elledge, 2007). Thus, as expected, the IRIF of γH2AX, MDC1, RNF8 and RNF168 were intact in *Rad6A*$^{-/-}$ and *Rad6B*$^{-/-}$ cells (Fig. 3D). However, the IRIF of BRCA1 and 53BP1 were significantly impaired in *Rad6A*$^{-/-}$, *Rad6B*$^{-/-}$ cells, or RAD6A and RAD6B double-depleted cells (Fig. 3D; supplementary material Figs S4, S5). These cellular phenotypes are very similar to those observed in RNF168-deficient cells (Doil et al., 2009; Stewart et al., 2009). Thus, these data support the hypothesis that RNF168 is the E3 ligase partner of RAD6A and 6B in the DNA-damage-induced ubiquitination cascade. It has been reported that RAD18 associates with 53BP1 and regulates the accumulation of 53BP1 in G1 cells (Watanabe et al., 2009). We examined the 53BP1 foci formation in RAD6A and 6B depleted cells released from thymidine block. As shown in supplementary material Fig. S5, the reduction of 53BP1 foci formation in RAD6A and 6B depletion cells was cell cycle independent. Thus, the results suggest that RAD6A and 6B play a broader role in the 53BP1 foci formation than RAD18.

Following DNA damage, RNF168-dependent ubiquitination events control the localization of BRCA1 and 53BP1, which regulate cell cycle checkpoint activation and DNA damage repair (DiTullio et al., 2002; Fernandez-Capetillo et al., 2002; Moynahan et al., 1999; Scully et al., 1997a; Scully et al., 1997b; Wang et al., 2002; Yarden et al., 2002; Yu and Chen, 2004). To study the functional significance of RAD6A and 6B during DNA damage response, we first examined whether RAD6A and 6B, like RNF168, regulate DNA-damage-induced checkpoint activation. Different from the IRIF observation, we did not observe any clear defects in *Rad6A*$^{-/-}$ or *Rad6B*$^{-/-}$ cells. This is likely a result of partial function redundancy, because RAD6A and 6B are homologs and may compensate for each other. Unfortunately, genetic deletion of both RAD6A and 6B induces cell lethality (Baarends et al., 2003). Thus, we used siRNA to knockdown both RAD6A and 6B. In response to DSBs, a Chk1-dependent checkpoint is activated and arrests cells at the G2/M transition (Liu et al., 2000; Lopez-Girona et al., 2001; Zhao and Piwnica-Worms, 2001). To examine the role of RAD6A and 6B in G2/M checkpoint activation, we examined the activation of Chk1 (marked by Chk1-Ser345 phosphorylation) in response to DSBs (Liu et al., 2000; Lopez-Girona et al., 2001; Zhao and Piwnica-Worms, 2001). Depletion of RAD6A and 6B abrogated DNA-damage-induced Chk1 activation (Fig. 4A). Correspondingly, IR-induced G2/M arrest was impaired in the absence of RAD6A and 6B (Fig. 4B), indicating that RAD6A and 6B are important in activating the G2/M checkpoint and suppress the G2/M transition when DSBs occur. To examine the role of RAD6A and 6B in homologous recombination (HR) repair, we examined the IRIF of Rad51, the effector of HR repair in the S phase. As shown in Fig. 4C, depletion of RAD6A and 6B significantly affected the IRIF of Rad51. Using an established GFP reporter assay to monitor HR repair (Pierce et al., 1999), we found that the loss of RAD6A and 6B negatively affected HR repair (Fig. 4D). Moreover, by using comet assay under neutral conditions, we also found that depletion of RAD6A and 6B increased DNA damage following IR treatment. As shown in Fig. 4E, the average comet tail moment in RAD6A and RAD6B double knockdown cells was much higher than that of control or single knockdown cells. In addition, cells lacking RAD6A and RAD6B were hypersensitive to IR (Fig. 4F). These data indicate that, like RNF168, RAD6A and 6B are important for DNA-damage-induced checkpoint activation and DNA damage repair.

To identify the potential targets of the RNF168/RAD6 complex, we performed *in vitro* ubiquitination assay by using different histones as substrates. Without any E3 ligase, RAD6 could weakly ubiquitinate most histones *in vitro* (Fig. 5A and supplementary material Fig. S6), which is consistent with previous reports (Kim and Roeder, 2009). Using recombinant RNF168 purified from sf9 cells, we found the RNF168/RAD6 complex can multi-mono-ubiquitinate histone H3 and H4 *in vitro* (supplementary material Fig. S6). However, we could not detect ubiquitinated histone H3 and H4 *in vivo* after IR treatment. Interestingly, we found the RNF168/RAD6 complex could promote the mono-ubiquitination of histone H1.2 *in vitro* (Fig. 5A). It is reported that RAD6 can act with the RNF20/RNF40 complex to mediate ubiquitination of H2B (Kim et al., 2009; Kim and Roeder, 2009; Moyal et al., 2011; Nakamura et al., 2011; Zhang and Yu, 2011). We did the *in vitro* ubiquitination of H1.2 by RAD6 along with the RNF20/RNF40/WAC complex (Zhang and Yu, 2011). However, the RNF20/RNF40/WAC complex could not promote H1.2 ubiquitination, neither did RNF168 induce H2B ubiquitination *in vitro* (Fig. 5B,C), suggesting that RAD6 mediates different histone ubiquitination through different E3 partners.

Moreover, we also found that H1.2 mono-ubiquitination can be induced by IR treatment (Fig. 5D). And the DNA-damage-induced H1.2 mono-ubiquitination is RAD6 and RNF168 dependent (Fig. 5E). Collectively, histone H1.2 is one of the

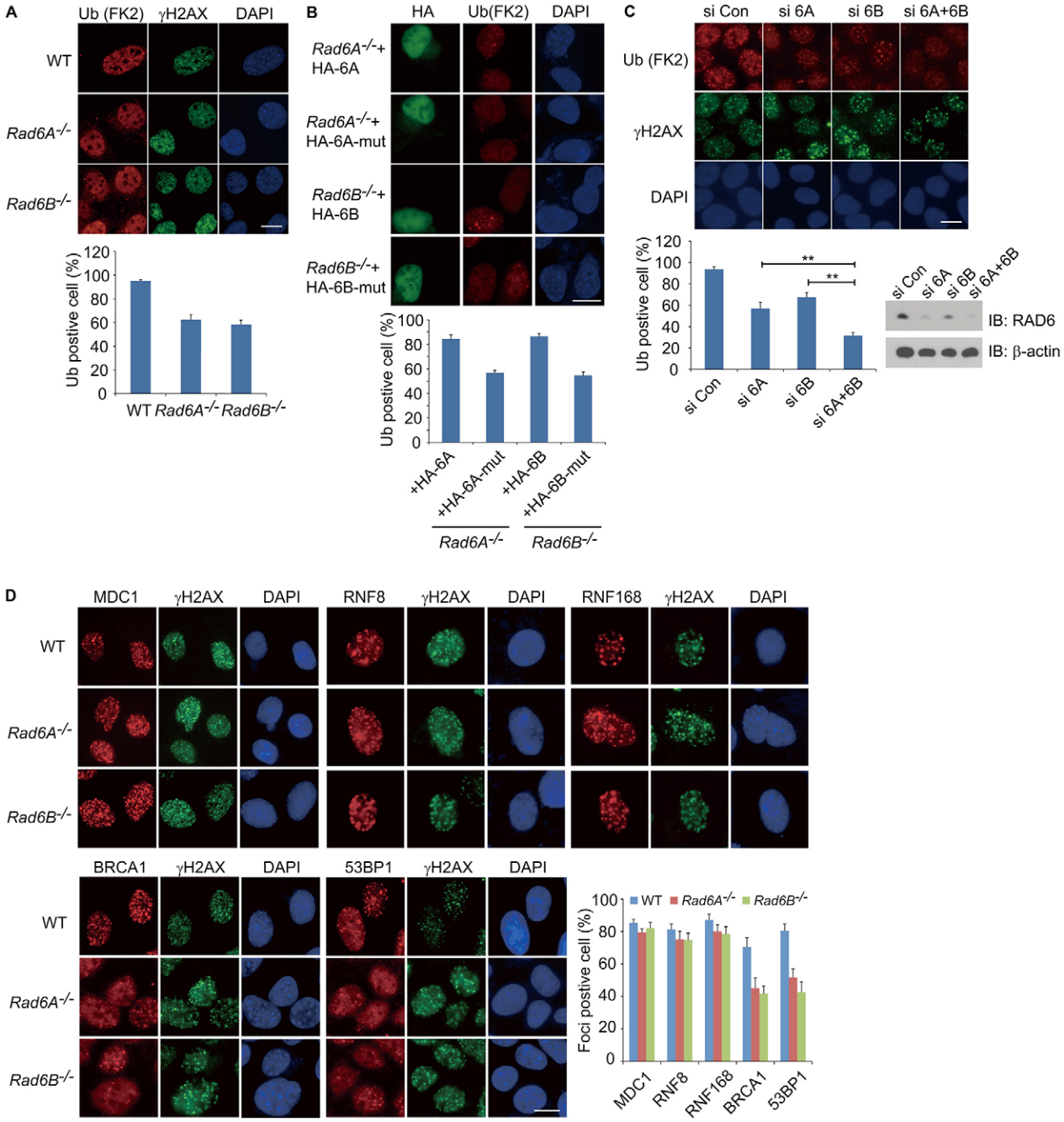

Fig. 3. RAD6A and RAD6B regulate IRIF of ubiquitin and other DNA damage repair factors. (**A**) RAD6A and 6B regulate ubiquitin foci formation at DNA damage sites. Wild-type (WT), *Rad6A*$^{-/-}$ or *Rad6B*$^{-/-}$ MEFs were treated with 3 Gy of IR. One hour later, cells were immunostained with monoclonal anti-Ub (FK2) and polyclonal anti-γH2AX antibodies. Cells with more than 10 foci in each nucleus were considered as foci-positive cells. The percentage of ubiquitin foci-positive cells in each MEF are summarized in the lower panel (mean±s.d.). At least 200 cells were counted in each sample and data were from three independent experiments. (**B**) Wild-type, but not mutated RAD6A or 6B, can restore the ubiquitin foci formation in RAD6A- or RAD6B-deficient cells following IR treatment. *Rad6A*$^{-/-}$ or *Rad6B*$^{-/-}$ MEFs were transfected with wild-type or mutated HA-tagged RAD6A or 6B (Pro64, Asn65 and Pro68 mutated to Ala) plasmids. After 24 hours, MEFs were treated with 3 Gy of IR. Ubiquitin foci and the expression of HA-tagged proteins were then detected by monoclonal anti-Ub (FK2) and polyclonal anti-HA antibodies. The percentage of ubiquitin-positive cells in HA-tagged protein-expressing cells are summarized in the lower panel (mean±s.d.). (**C**) RAD6A and 6B have partially redundant roles in regulating ubiquitin IRIF. RAD6A or 6B were single- or double-depleted in U2OS cells by siRNA treatment. After 48 hours, cells were treated with 3 Gy of IR and the IRIF of ubiquitin was examined 1 hour after irradiation (mean±s.d., $^{**}P<0.01$.). si Con, control siRNA; si 6A, RAD6A siRNA; si 6B, RAD6B siRNA; si 6A+6B, RAD6A and RAD6B siRNAs. (**D**) RAD6A and 6B regulate the IRIF of BRCA1 and 53BP1, but not γH2AX, MDC1, RNF8 or RNF168. Wild-type, *Rad6A*$^{-/-}$ and *Rad6B*$^{-/-}$ MEFs were treated with 10 Gy of IR. One hour later, cells were fixed and immunostained with the indicated proteins. FLAG-RNF8 and FLAG-RNF168 were transfected into MEFs 24 hours before IR treatment and IRIF were immunostained with monoclonal anti-FLAG antibody. The percentage of foci-positive cells for each protein are summarized in the histogram (mean±s.d., data from three independent experiments). Scale bars: 20 μm.

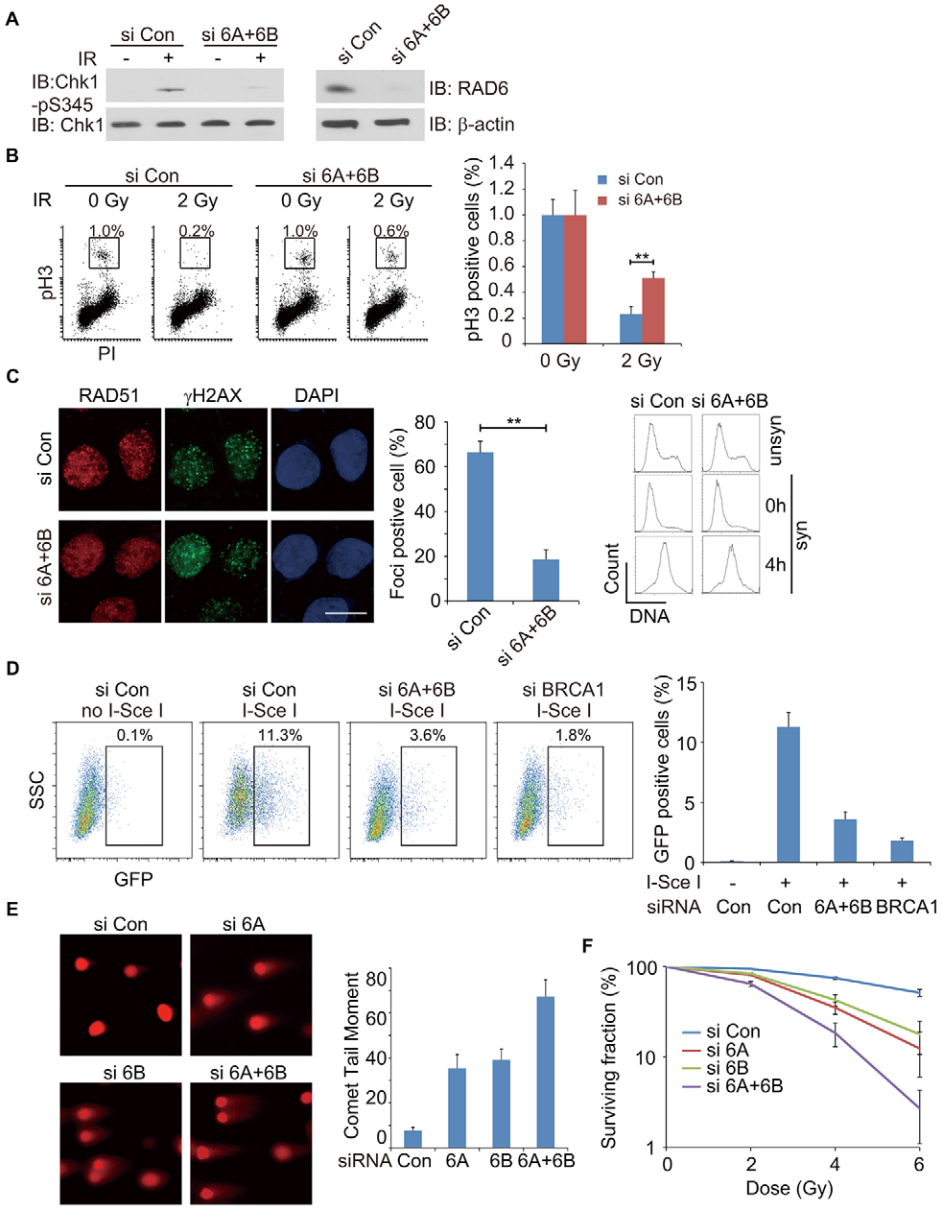

Fig. 4. RAD6A and RAD6B are required for G2/M checkpoint activation and DNA damage repair. (**A**) Double knockdown of RAD6A and 6B disrupts IR-induced Chk1 activation. U2OS cells were transfected with the indicated siRNAs. After 48 hours, cells were irradiated with 10 Gy of IR. One hour later, Chk1 activation was analyzed by western blot using anti-Chk1 pSer345 and anti-Chk1 antibodies. The expression of RAD6 after siRNA treatment is shown on the right. (**B**) RAD6A and 6B participate in DNA-damage-induced G2/M checkpoint activation. U2OS cells were transfected with the indicated siRNAs. After 48 hours, cells were irradiated with or without 2 Gy of IR. One hour later, cells were harvested and stained with anti-histone H3 pSer10 antibody. The mitotic population was examined by flow cytometry analysis. Data were from three independent experiments (mean±s.d.). (**C**) Depletion of RAD6A and 6B impairs the IRIF of Rad51 at S phase. U2OS cells were treated with the indicated siRNA and synchronized by thymidine block for 20 hours. Then, cells were released with fresh medium for 4 hours and irradiated with 3 Gy of IR. One hour later, cells were stained with polyclonal anti-RAD51 and monoclonal γH2AX antibodies. The percentage of foci-positive cells is summarized in the histogram (mean±s.d.). Cell cycle profiles are shown on the right. Unsynchronized cell cycle profile is shown as control; unsyn, unsynchronized; syn, synchronized. (**D**) Depletion of RAD6A and 6B impairs HR repair. U2OS-DrGFP cells were treated with the indicated siRNAs for 24 hours. Cells were then infected with adeno-I-SecI virus. After 48 hours, cells were harvested and HR repair was examined by FACS analysis. The percentage of GFP-positive cells is summarized on the right. Data (mean±s.d.) were from three independent experiments. (**E**) Representative microphotographs of comet assay from cells treated with the indicated siRNAs. The comet tail moments were analyzed from at least 100 cells in each sample with CometScore software. Data are presented as mean±s.e.m. from three independent experiments. (**F**) U2OS cells lacking RAD6A and 6B are sensitive to IR. U2OS cells treated with the indicated siRNAs were subjected to colony formation assay. Data are presented as mean±s.d. from three independent experiments. si Con, control siRNA; si 6A, RAD6A siRNA; si 6B, RAD6B siRNA; si 6A+6B, RAD6A and RAD6B siRNAs; si BRCA1, BRCA1 siRNA. Scale bar: 20 μm. $^{**}P<0.01$.

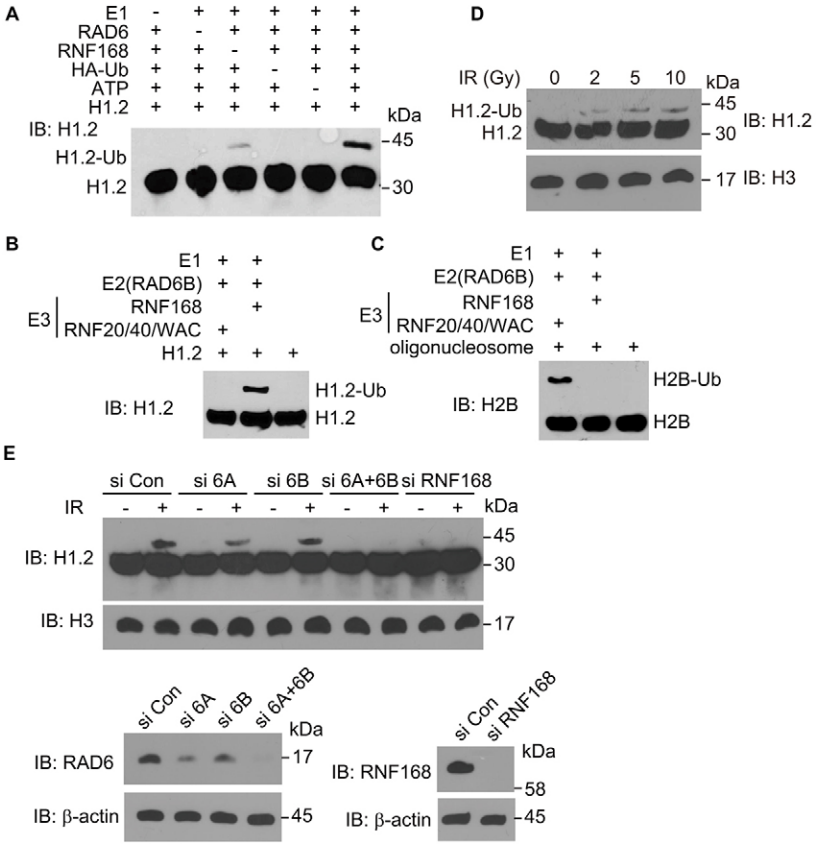

Fig. 5. RNF168–RAD6 complex ubiquitinates histone H1.2 *in vitro* and regulates ubH1.2 *in vivo*. (**A**) RNF168–RAD6B ubiquitinates histone H1.2 *in vitro*. *In vitro* ubiquitination assay was performed as described in Materials and Methods. Reaction mixtures were then subjected to SDS-PAGE and western blot with anti-H1.2 antibody. (**B**) *In vitro* H1.2 ubiquitination assay was performed with H1.2, E1, RAD6B, RNF168 or the RNF20–RNF40–WAC complex, and HA-Ub. Products were resolved by SDS-PAGE and blotted with anti-H1.2 antibody. (**C**) *In vitro* H2B ubiquitination assay were performed in the presence of oligonucleosome, E1, RAD6B, RNF168 or the RNF20–RNF40–WAC complex and HA-Ub. Products were then resolved by SDS-PAGE and blotted with anti-H2B antibody. (**D**) Histone H1.2 is mono-ubiquitinated following IR treatment. 293T cells were irradiated with the indicated doses of IR. One hour later, the chromatin fraction of each sample was subjected to SDS-PAGE and blotted with anti-H1.2 antibody. Histone H3 was loading control. (**E**) RNF168–RAD6 regulates ubH1.2 *in vivo*. U2OS cells were transfected with the indicated siRNAs. After 48 hours, cells were irradiated with 30 Gy of IR. One hour later, histone H1.2 in the chromatin fraction was examined by western blot. Histone H3 was used as the protein loading control. The expression of each protein after siRNA treatment is shown in the lower panel. si Con, control siRNA; si 6A, RAD6A siRNA; si 6B, RAD6B siRNA; si 6A+6B, RAD6A and RAD6B siRNAs; si RNF168, RNF168 siRNA.

physiological relevant substrates of the RNF168/RAD6 complex in response to DNA damage.

Discussion

Identification of RNF168 by Steward et al. and Doli et al. demonstrated the functional significance of RNF168 in response to DNA damage. In this study, we present extensive evidence that RAD6A and RAD6B are functional partners of RNF168. This implicates the role of RNF168 is in a more complicated ubiquitination cascade than previously thought. Following DNA damage, the RNF8/Ubc13 complex is recruited to DNA damage sites by phospho-MDC1 to initiate ubiquitin signals on histones H2A and H2B (Huen et al., 2007; Kolas et al., 2007; Mailand et al., 2007; Wang and Elledge, 2007). The ubiquitin binding motifs of RNF168 recognize these initial ubiquitin events (Doil et al., 2009; Stewart et al., 2009) and may target RAD6A and 6B to DNA damage sites via the RNF168 RING domain to catalyze the ubiquitination of histones H1.2, one of its substrates at the DNA damage sites. Our study establishes, for the first time, that H1.2 is mono-ubiquitinated in response to IR treatment. Since H1.2 ubiquitination is also controlled by RNF8 and Ubc13 *in vivo*, but not directly ubiquitinated by the RNF8/Ubc13 complex *in vitro* (supplementary material Fig. S7), it is likely that H1.2 ubiquitination is a downstream event of initial IR-induced protein ubiquitination catalyzed by the RNF8/Ubc13 complex.

Recent evidence indicates that H2B ubiquitination may significantly alter the topology of the chromatin and induce chromatin relaxation (Fierz et al., 2011; Moyal et al., 2011). Thus, the RNF168/RAD6-dependent H1.2 ubiquitination is also likely to loosen chromatin fibers and facilitates the recruitment of other DNA damage response factors, including other E3 ligases to the DNA damage sites. Although RNF168/RAD6 only promote mono-ubiquitination of H1.2 *in vitro* and *in vivo*, it is still likely the mono-ubiquitination may set up a proper micro-environment for other E2/E3 complexes to catalyze poly-ubiquitination or multi-mono-ubiquitination of H2A and H2AX on top of RNF8/Ubc13-dependent ubiquitination of H2A and H2AX. Specifically, it has been reported that the RNF168/Ubc13 and RNF168/UbcH5C complex could catalyze multi-ubiquitination on H2A and H2AX (Doil et al., 2009; Mattiroli et al., 2012; Stewart et al., 2009). Here, after depleting RAD6 and 6B by siRNA treatment, the IR-induced multi-ubiquitination of H2A and H2AX was suppressed (supplementary material Fig. S8). Thus, it is possible that the RNF168/RAD6 complex applies the initial ubiquitin on H1.2. The MIU motifs of RNF168 may recognize ubH1.2, and induce the morphology changes in RNF168, which might be important for association with Ubc13 or UbcH5C. These RNF168 complexes could then further multi-ubiquitinate H2A and H2AX during DNA damage response. Although in a pulldown assay the affinity between RNF168 and Ubc13/Mms12 was too weak to be detected in the absence of ubiquitin (Fig. 2A and supplementary material Fig. S9), it is still possible that mono-ubiquitinated substrates could induce RNF168/Ubc13 complex formation for the sequential ubiquitination on H2A and H2AX. Further structural analysis of the RNF168 complex will test these possibilities.

We also realize that besides histone H1.2, RNF168/RAD6 may ubiquitinate other substrates at DNA damage sites, which could be important for DNA damage response. Further searching for the physiologically relevant substrates will reveal the function

and molecular mechanism of the DNA-damage-induced ubiquitination cascade. Besides RNF168, both RAD18 and the RNF20/RNF40/WAC complex are known partners of RAD6A and 6B (Kim et al., 2009; Lawrence, 2007; Prakash et al., 2005). RAD6A and 6B are evolutionarily conserved, and have a common yeast ortholog. Genetic screening in *Saccharomyces cerevisiae* demonstrates that yeast RAD6 is the enzymatic partner of RAD18, which is required for the ubiquitination of PCNA following DNA damage and facilitates translesion synthesis to bypass DNA damage sites during replication (Haracska et al., 2006; Hoege et al., 2002). In addition, a subset of RAD6 forms a complex with yeast Bre1 and its mammalian homolog RNF20/RNF40 through a coiled-coil region, but not the RING domain (Kim et al., 2009). The complex of RNF20/RNF40/RAD6 also contributes to the DNA damage response, particularly during HR repair (Moyal et al., 2011; Nakamura et al., 2011). However, RNF20 is not required for DNA-damage-induced ubiquitin foci formation. Thus, RNF20/RNF40/RAD6 could be the downstream E2/E3 complex for the amplification of the ubiquitin signals at DNA damage sites or form an H2AX-independent pathway at DNA damage sites (Lawrence, 2007; Moyal et al., 2011; Nakamura et al., 2011). Since RNF168 and RNF20 are associated with RAD6 and involved in HR repair, it is possible that both the RNF168/RAD6 and RNF20/RNF40/RAD6 complex participate in HR repair, but at different stages. Further studies will be necessary to determine what factors are involved to fully understand the ubiquitination cascade that occurs in response to DNA damage.

Both *Rad6A* and *Rad6B* knockout mice have been generated (Roest et al., 2004; Roest et al., 1996). Single gene knockout mice are viable, whereas knockout of both *RAD6A* and *RAD6B* causes early embryonic lethality (Roest et al., 2004; Roest et al., 1996). Interestingly, although *Rad6B*$^{-/-}$ mice are viable, the male mice are sterile (Roest et al., 1996). Detailed analyses revealed that spermatogenesis is arrested during the last few steps of sperm maturation when nucleosomal histones need to be replaced by histone-like proteins (Baarends et al., 2003). This spermatogenesis defect is very similar to that observed in *Rnf8*$^{-/-}$ mice (Li et al., 2010; Lu et al., 2010; Santos et al., 2010), suggesting that RAD6 functions in the RNF8-dependent pathway at specific points in tissue and organ development, which might mimic the biological processes observed during DNA damage response.

Materials and Methods

Plasmids, antibodies and other materials

Full length RNF168, RNF8, RNF168-I18A and 30 E2 enzymes were cloned into the SBP vector (S-FLAG-SBP-tagged). RAD6A, RAD6B, Ubc13, RAD6A/B-mut (P64A, N65A and P68A) were cloned into pCMV-HA vector. RAD6A, RAD6B, Ubc13 and RAD6A/B-mut (P64A, N65A and P68A) were cloned into the pGEX-4T-1 vector (Amersham) to generate glutathione S-transferase (GST) fusion proteins.

Polyclonal anti-mouse BRCA1, anti-RNF8, anti-53BP1 antibodies, polyclonal anti-human BRCA1, anti-γH2AX antibodies and anti-RAD51 antibody were previously described (Wu et al., 2009). Anti-ubiquitin (FK2), anti-histone H3 and anti-phospho-histone H3 (pSer10) were purchased from Upstate. Anti-GST, anti-HA, anti-FLAG and anti-β-actin antibodies were purchased from Sigma. Anti-RAD18, anti-H1.2 antibodies were purchased from Proteintech and Covalab, respectively. Anti-Chk1 and phospho-Chk1 (pSer345) antibodies were purchased from Cell Signaling Technology. Anti-K63 and K48-linked poly-Ub antibodies were purchased from Genentech. Anti-human RAD6 antibody was raised against GST-human RAD6B (full length). Since human RAD6A and RAD6B share 96% homology, anti-human RAD6 antibody recognizes both RAD6A and RAD6B. Anti-RNF168 antibody was raised against GST-human RNF168-C terminus (amino acids 301–571).

The siRNA duplexes were purchased from Dharmacon Research (Lafayette, CO). The siRNA sequences targeting RAD6A and RAD6B are GAACAAGCUGGCGUGAUUdTdT, and CAAACGAGAAUAUGAGAAAdTdT, respectively. The siRNA sequences targeting RNF168 and RAD18 were CCUUGGAGUUGGAGAACAAUU and ACUCAGUGUCCAACUUGCUdTdT, respectively. The siRNA sequences targeting BRCA1 was GGAACCUGUCTCCACAAAGdTdT. The siRNA sequences targeting RNF8 was AGAAUGAGCUCCAAUGUAUUU, and the siRNA sequences targeting Ubc13 was GCACAGUUCUGCUAUCGAUUU. siRNAs were transfected into the cells using oligofectamine (Invitrogen) according to the manufacturer's instructions.

Cell culture and treatment with ionizing radiation

293T, U2OS cells and MEFs were cultured in the Dulbecco's modified Eagle medium with 10% fetal bovine serum. *Rad6A*$^{-/-}$ and *Rad6B*$^{-/-}$ MEFs were gifts from Dr Hoeijmakers (Erasmus University). For IR treatment, cells were irradiated using a JL Shepherd 137Cs radiation source with the indicated doses and then recovered in the same culture conditions for further analysis.

Immunofluorescence staining

After IR treatment and recovered for 1 hour unless specifically indicated, cells grown on coverslips were fixed in 3% paraformaldehyde for 5 minutes and permeabilized with 0.5% Triton X-100 in phosphate-buffered saline (PBS) for 5 minutes at room temperature. Samples were blocked with 8% goat serum for 30 minutes and then incubated with the primary antibodies for 1 hour. After washed with PBS three times, samples were incubated with the secondary antibodies for 30 minutes. Nuclei were then counterstained with DAPI. After a final wash with PBS, coverslips were mounted with glycerin containing p-phenylenediamine. All images were obtained with an OLYMPUS IX71 fluorescence microscope. In the IRIF analysis, cells with more than 10 foci in each nucleus were considered as foci positive cells, since spontaneous foci exist in untreated cells. For the RAD6 rescue experiment, cells were transfected with the indicated plasmids 24 hours before the IR treatment.

Laser microirradiation

U2OS cells were grown on 35-mm glass bottom dishes (MatTek Corporation) and treated with the indicated siRNAs for 48 hours. Cells were then subjected to laser microirradiation. Laser microirradiation was carried out on an OLYMPUS IX71 inverted fluorescence microscope with a Micropoint® Laser Illumination and Ablation System (Photonic Instruments). 30 minutes later, cells were subjected to immunostaining with the indicated antibodies.

Cell lysis, GST pull-down assay and western blotting

Cells were lysed with the NETN buffer (0.5% NP-40, 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, and 100 mM NaCl) on ice for 10 minutes. Soluble fraction was then subjected to further analysis. GST pull-down assay and western blotting were performed following standard protocols.

Chromatin fraction

Cells were harvested at indicated time point after IR treatment, and washed twice with PBS. Cell pellets were subsequently resuspended in the NETN buffer, and incubated on ice for 10 minutes. Insoluble fraction was recovered and resuspended in 0.2 M HCl for 10 minutes at room temperature. The soluble fraction of HCl extraction was neutralized with 1 M Tris-HCl (pH 8.0) for further analysis.

DSP crosslinking and immunoprecipitation

293T cells were collected and washed three times with PBS 30 minutes after 10 Gy of IR treatment. DSP was then added to a final concentration of 2 mM. After incubation for 2 hours on ice, 1 M Tris-HCl (pH 7.5) was added to stop the reaction. Cells were collected and lysed with the NETN300 buffer (0.5% NP-40, 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, and 300 mM NaCl). Soluble fraction was subjected to standard immunoprecipitation process.

In vitro ubiquitination assay

RNF168, RNF20/RNF40, and RAD6B were cloned into the pFastBac vector. Recombinant proteins from sf9 cells were purified by a Hiload Superdex 200 column and UNO Q-1 column using Bio-Rad Duo Flow system. HA-ubiquitin, E1, and Ubc13/Uev1a enzyme were purchased from Boston Biochem. Recombinant GST-H1.2 was expressed and purified from *E. Coli*. BL21(DE3). H1.2 protein was collected by thrombin (Sigma) cleavage. *In vitro* chromatin assembly was performed according to manufacturer's instruction (Active Motif). *In vitro* ubiquitination assays were carried out with 50 ng E1, 100 ng E2, 300 ng E3, 500 ng histones or chromatin generated from 0.2 μg input DNA and 1.5 μg HA-ubiquitin in 20 μl Ub assay buffer (50 mM Tris-HCl pH 7.5, 5 mM MgCl2, 2 mM NaF, 0.5 mM DTT, 2 mM ATP) at 30℃ for 1 hour. The resulting products were resolved by SDS-PAGE and subjected to western blot.

G2/M checkpoint assay

U2OS cells were transfected with control or RAD6A and 6B siRNAs for 48 hours. The cells were treated with or without 2 Gy of IR. One hour later, cells were fixed with 70% ethanol and stained with anti-phospho-histone H3 antibody (pH 3), followed by incubation with fluorescein isothiocyanate-conjugated secondary antibody. The stained cells were treated with RNase, incubated with propidium iodide, and then analyzed by flow cytometry.

Homologous recombination repair assay

U2OS cells with a single copy of DR-GFP were treated with control, BRCA1, RAD6A and 6B siRNAs. 24 hours later, cells were infected with adenovirus that expresses I-SceI (adeno-I-SceI). After 48 hours, cells were subjected to flow cytometry analysis to examine homologous recombination.

Thymidine block and cell cycle analysis

Cells were arrested in 2 mM thymidine for 20 hours and released in fresh medium for indicated time. The cells were collected for cell cycle profile analysis by propidium iodide staining.

Neutral comet assays

Single-cell gel electrophoretic comet assays were performed under neutral conditions. U2OS cells were first treated with control or RAD6A and 6B siRNA and blocked by thymidine treatment as described above before IR treatment. The cells were irradiated with 25 Gy of IR and recovered in normal culture medium for 3 hours. Cells were collected and rinsed twice with ice cold PBS. 1×10^5 cells/ml were combined with LMAgarose at 37℃ at a ratio of 1: 10 (v/v) and immediately pipetted onto slides. For cell lysis, the slides were immersed in the neutral lysis solution (2% sarkosyl, 0.5 M EDTA, 0.5 mg/ml proteinase K, pH 8.0) overnight at 37℃. Then, the slides were subjected to electrophoresis at 15 V for 25 minutes (0.6 V/cm), and stained in 10 g/ml propidium iodide for 20 minutes. Images were taken with a fluorescence microscope and analyzed with the CometScore software.

Colony formation assay

U2OS cells were treated with control or RAD6A and 6B siRNAs. One thousand cells were plated in the wells of a 6-well plate immediately following irradiation. After incubation for 10 days, the surviving cell fractions were calculated by comparing the number of colonies formed in the irradiated cultures with those in mock treated control.

Statistical analysis

Data were analyzed by the Student's *t* test with $P<0.05$ considered to be significant.

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Author contributions

C.L. and D.W. performed the experiments, analyzed data and participated in the writing of the manuscript. J.W., J.K. and T.M. performed the experiments. X.Y. designed experiments and wrote the manuscript.

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