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RNF168 Ubiquitinates K13-15 on H2A/H2AX to Drive DNA Damage Signaling

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SUMMARY

Ubiquitin-dependent signaling during the DNA damage response (DDR) to double-strand breaks (DSBs) is initiated by two E3 ligases, RNF8 and RNF168, targeting histone H2A and H2AX. RNF8 is the first ligase recruited to the damage site, and RNF168 follows RNF8-dependent ubiquitination. This suggests that RNF8 initiates H2A/H2AX ubiquitination with K63-linked ubiquitin chains and RNF168 extends them. Here, we show that RNF8 is inactive toward nucleosomal H2A, whereas RNF168 catalyzes the monoubiquitination of the histones specifically on K13-15. Structure-based mutagenesis of RNF8 and RNF168 RING domains shows that a charged residue determines whether nucleosomal proteins are recognized. We find that K63 ubiquitin chains are conjugated to RNF168-dependent H2A/H2AX monoubiquitination at K13-15 and not on K118-119. Using a mutant of RNF168 unable to target histones but still catalyzing ubiquitin chains at DSBs, we show that ubiquitin chains per se are insufficient for signaling, but RNF168 target ubiquitination is required for DDR.

INTRODUCTION

Ubiquitination of target proteins was discovered by the identification of this mark on histone 2A (H2A) at lysine 119 (Goldknopf and Busch, 1977; Hunt and Dayhoff, 1977). This prevalent modification can be present at up to 10% of cellular H2A in chromatin as a result of ubiquitination by the E3 ligases present in the Polycomb repressive complex 1 (PRC1) during transcriptional repression (Buchwald et al., 2006; Cao et al., 2005; Li et al., 2006; Wang et al., 2004). More recently it was suggested that two RING-type ubiquitin E3 ligases, RNF8 and RNF168, with roles in the DNA damage response (DDR) also modify H2A (Doil et al., 2009; Huen et al., 2007; Kolas et al., 2007; Mailand et al., 2007; Marteijn et al., 2009; Pinato et al., 2009; Stewart et al., 2009; Wang and Elledge, 2007).

These ligases participate in the early signaling events of the double-strand break (DSB) repair pathway, where their ubiquitination activity is required for proper DDR. RNF8 is the first ligase that binds to damaged sites in an ATM-dependent manner (Huen et al., 2007; Kolas et al., 2007; Mailand et al., 2007; Wang and Elledge, 2007). The E3 ligase activity of RNF8, conferred by its C-terminal RING domain, is required for the formation of ubiquitin chains at the site of damage to permit the recruitment of the second E3 ligase, RNF168, by its ubiquitin binding domains (Doil et al., 2009; Pinato et al., 2009; Stewart et al., 2009). The concerted action of these ligases contributes to the ubiquitin-dependent signals necessary for the further recruitment of downstream regulators of the DSB response pathway, such as 53BP1 and the BRCA1 complex (Al-Hakim et al., 2010; Kim et al., 2007; Panier and Durocher, 2009; Sobhian et al., 2007; Wang et al., 2002; Yan et al., 2007).

Very little is known about the substrate spectrum of the ligase activity of RNF8 and RNF168 during DDR. So far the main targets are histone H2A and its variant H2AX, where the E3s are thought to form K63-linked ubiquitin chains (Doil et al., 2009; Huen et al., 2007; Kolas et al., 2007; Mailand et al., 2007; Pinato et al., 2009; Stewart et al., 2009; Wang and Elledge, 2007). Because of their recruitment order, it has been proposed that RNF8 is the ligase responsible for the initial ubiquitination of the histones, while RNF168 is thought to extend the chains on this target.

More recent data have revealed higher complexity to this pathway, where phosphorylation and sumoylation are signaling in conjunction with the ubiquitination-dependent cascade (Bekker-Jensen and Mailand, 2011; Bensimon et al., 2011; Stucki and Jackson, 2006). Additional ubiquitin ligases also participate in the damage response, such as the BRCA1/BARD1 complex that is recruited by the actions of RNF8 and RNF168 and is responsible for downstream signaling (Kim et al., 2007; Sobhian et al., 2007; Yan et al., 2007), the HECT-type E3 HERC2 that is recruited by RNF8 in a phosphorylation dependent manner and appears to have different substrate spectrum than the

RING-type ligases RNF8 and RNF168 (Bekker-Jensen et al., 2010), RAD18 and the negative regulator RNF169 whose involvement in DSB repair was recently described (Huang et al., 2009; Poulsen et al., 2012). Also the Polycomb Ring1B/Bmi1 complex is implicated in the DSB response, where its activity toward K119 of histone H2A appears to be important (Bergink et al., 2006; Chagraoui et al., 2011; Facchino et al., 2010; Ginjala et al., 2011; Ismail et al., 2010; Pan et al., 2011; Wu et al., 2011). In this complex scenario, the current literature lacks biochemical details to clarify the molecular events that define the DNA damage-dependent H2A/H2AX ubiquitination orchestrated by RNF8 and RNF168.

Here, we study the mechanism of RNF168 and RNF8. Surprisingly we find that the first step of H2A ubiquitination is catalyzed by RNF168, while RNF8 is inactive toward this target. To confirm this finding, we solve the structure of the catalytic RING domain of RNF8 to find the region that is responsible for target specificity for these ligases. Using specific single-point target recognition mutants we validate the importance of this region on RING domains for the function of these E3s in vitro and in vivo.

We find that RNF168-dependent H2A ubiquitination during DDR takes place at a previously unknown site on H2A, on K13 or 15. We also show that K63 ubiquitin chains are specifically formed on this site and not on the Polycomb-modified residue. We find that RNF8 is efficient in catalyzing these chains in vitro on the already ubiquitinated histone. Interestingly we show that it is this target ubiquitination step by RNF168 that is critical for the integrity of the DDR pathway.

RESULTS

RNF168 Efficiently Modifies H2A in Nucleosomes In Vitro, and RNF8 Is Inactive toward this Target

In order to understand the molecular details of the early steps of the DSB repair pathway, we reconstituted in vitro the reactions carried out by RNF8 and RNF168 toward H2A using purified human oligonucleosomes as substrate.

We first assessed the intrinsic activity of these ligases in catalyzing the formation of ubiquitin chains in vitro in absence of the target. Purified full-length human RNF8 or RNF168, as well as their isolated RING domains (Figure 1A) were incubated with E1, Mg^{2+}, ATP, ubiquitin, and a set of E2s. The formation of ubiquitin chains was followed by western blot analysis and in these assays both E3 ligases were active with several E2s. RNF8 showed high activity particularly with UbcH5c and the Ubc13/Mms2 complex, depleting the pool of free ubiquitin (Figure 1B; Figure S1A available online). Although the activity of RNF168 was lower, it still promoted ubiquitin conjugation with these E2 enzymes (Figures 1B and S1A).

Previous studies have shown that both RNF8 and RNF168 are active toward isolated purified histone proteins (Doil et al., 2009; Mailand et al., 2007; Pinato et al., 2009). In our hands, both E3 ligases ubiquitinate H2A in its free form, or in presence of the other histones without DNA (Figure 1C). However on purified oligonucleosomes, where the histone octamer is surrounded by DNA only RNF168 maintains its capacity to target H2A, primarily with the E2 UbcH5c. In contrast, full-length RNF8 and its RING domain are extremely inefficient in modifying nucleosomal H2A with any of the E2s (Figures 1C, 1D, and S1C–S1F). Comparable results were obtained for histone H2AX (Figure 1E). Additionally, from the analysis of the other histone proteins in nucleosomes we see that RNF168 can target H2B, but not H3 and H4, to a lower extent than the H2A-type histones (Figure S1G) while RNF8 is inactive.

The finding that RNF168 targets H2A/H2AX efficiently, whereas RNF8 is inactive, contrasts with the order of recruitment of these ligases to the damage site, and thus challenges the current view on the order of events of H2A ubiquitination by RNF8 and RNF168 in the DDR.

Crystal Structure of the RING Domain of RNF8

RNF8 is capable of modifying H2A in isolation, but it is inactive when the histone is in its most common form within the nucleosome. We determined the crystal structure of the RING domain of RNF8 to understand the molecular determinants of this target specificity. The structure was solved at 1.9 Å resolution using the anomalous signal of the Zinc ions (Figure 2A), with R/Rfree of 20.0/22.6% and excellent stereochemistry (Table S1). The Zinc-binding region in the RING (403–441) forms the canonical structure with two Zn^{2+} ions coordinated by the C3HC4 motif. It resembles other RING structures, including the recently determined RNF168 monomer (PDB: 3L11, rmsd 2.0 Å for the monomer) (Figure 2B) (Campbell et al., 2012).

The RNF8 RING domain forms a dimer through interactions of the core RING domain and flanking regions in a manner that resembles other dimeric RING dimers such as Ring1B/Bmi1 (Buchwald et al., 2006; Li et al., 2006), BRCA1/BARD1 (Brzovic et al., 2001), RAD18 (Huang et al., 2011) and the U-box protein CHIP (Zhang et al., 2005) (Figure 2B), but with an extended buried interface (~2,000 $Å^2$), due to long N-terminal helices that fully contribute to the dimer interface, in accordance with the low resolution structure recently published (Campbell et al., 2012).

We used SAXS (small angle X-ray scattering) (Mertens and Svergun, 2010; Putnam et al., 2007) to study the shape of the RNF8 RING domain and confirmed the extended arrangement of these helices in solution. Ab initio modeling based on these SAXS measurements resulted in a molecular shape that fits the crystal structure well (Figures 2C and S2A).

In the crystal structure, these N-terminal helices adopt 35° different orientations in each protomer within the dimer, revealing the possibility of a structural asymmetry that was also observed in full-length CHIP (Zhang et al., 2005) (Figure 2D). Although most likely due to crystal contacts, the point of asymmetry is conserved between RNF8 and CHIP (Figure S2B). In general, structural and functional asymmetry seems to be a conserved feature in this class of E3-ligases, where, e.g., in Ring1B/Bmi1, BRCA1/BARD1, RAD18, and CHIP only one protomer is active (Brzovic et al., 2001; Buchwald et al., 2006; Hibbert et al., 2009; Huang et al., 2011; Masuda et al., 2012; Zhang et al., 2005).

Identification of a Target Recognition Site on RING Domains Explains the Differences in Activity toward H2A in Nucleosomes

We compared the crystal structure of RNF8, which is inactive against nucleosomal H2A, with crystal structures of two E3 ligases that can catalyze H2A monoubiquitination, RNF168 and

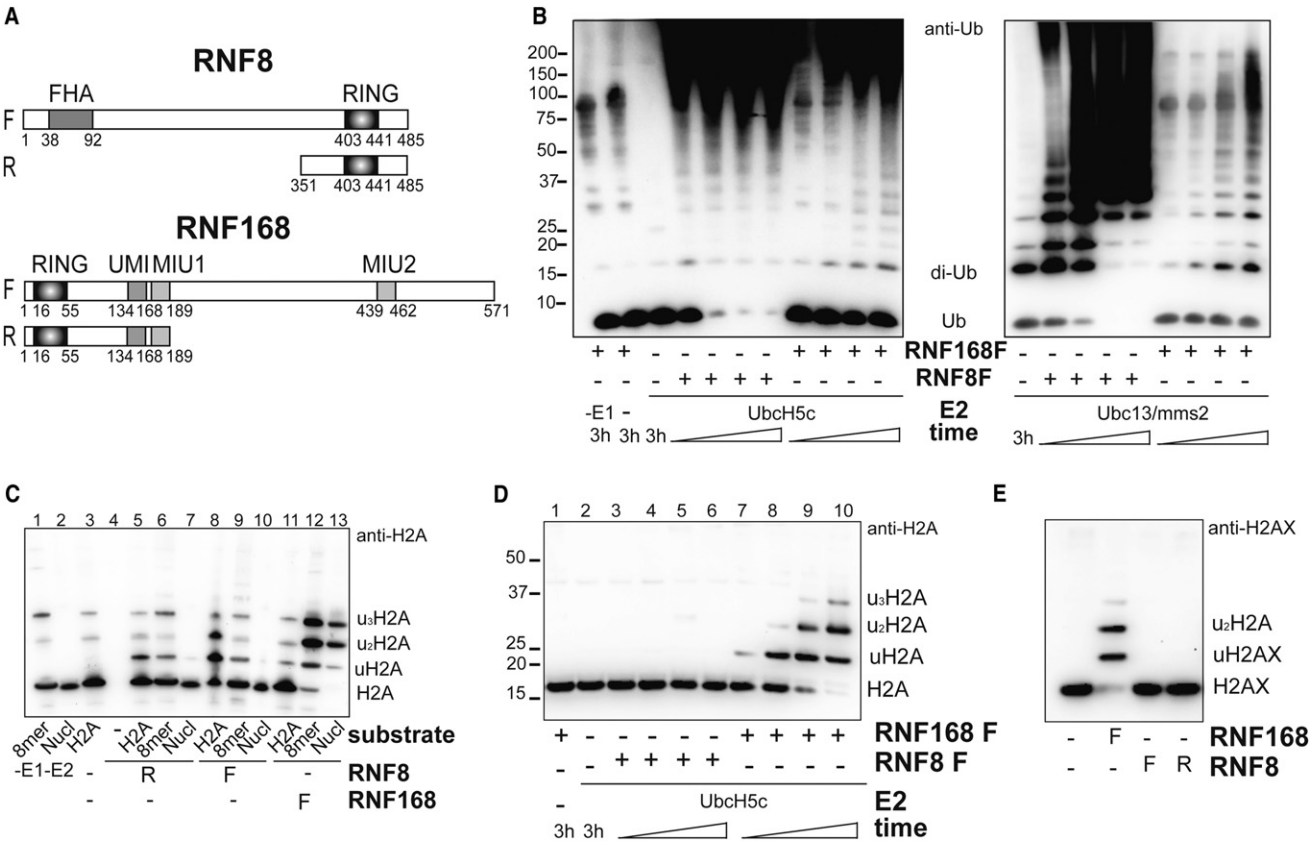

Figure 1. RNF168 Efficiently Modifies H2A in Nucleosomes In Vitro, and RNF8 Is Inactive toward this Target

(A) Domain architecture of human RNF8 and RNF168. Full-length (F) and RING (R) domain constructs used.

(B) Time course (10-30-90-180 min) ubiquitin chain formation assay of 1 μM full-length RNF8 and RNF168 with the E2 enzymes UbcH5c and Ubc13/Mms2 in presence of 15 μM ubiquitin.

(C) Full-length and RING domain of RNF8 can efficiently ubiquitinate isolated H2A or H2A in the context of the octamer (lanes 5 and 6 and 8 and 9), whereas they are inefficient in ubiquitinating H2A in the nucleosomal context (lane 7 and 10). RNF168 can efficiently target H2A in each context (lanes 11–13).

(D) H2A ubiquitination in purified oligonucleosomes (time course, as above) with 1 μM E3. Concentration series is shown in Figure S1E.

(E) RNF168 can modify H2AX in nucleosomes and RNF8 does not (3 hr).

Ring (R), full-length (F). See also Figure S1.

Ring1B. This allowed us to search for regions involved in substrate recognition in these E3s. When we superimposed the RING domains in these structures (Figure 3A) we noted a significant deviation located just C-terminal to the last cysteine of the RING motif, flanked by a charged residue that is negative in RNF8 (D443), but positively charged in RNF168 (R57) and Ring1B (K93).

We inverted the charges at this single site on both the RING domain and the full-length proteins to analyze the importance of this charged residue for the activity of the ligases.

Interestingly, a R57D mutation in RNF168, which does not affect the monomeric nature of the protein (Figure S3G), fully abolishes its ability to ubiquitinate H2A in nucleosomes (Figure 3B). Importantly, this single point mutation hardly affects the formation of ubiquitin chains by RNF168 (Figure 3C). It also does not affect the rate of discharging ubiquitin from the E2 enzyme UbcH5c, as shown in single turnover experiments on pre-charged UbcH5c∼ubiquitin (Figures 3D and S3A–S3C). Apparently this site is important for target specificity but not for ubiquitin chain formation.

On the other hand, the reverse change in charge at this site (D443R) in RNF8, confers the ability to the RING domain construct to target H2A in nucleosomes (Figure 3E, time course in Figure S3I). Again, the mutation has almost no effect on the ability to form unanchored ubiquitin chains nor on the rate of discharging the E2 (Figures 3F, 3G, and S3D–S3F). The gain-of-function, though present, is much less for the full-length RNF8 protein toward the H2A target, suggesting that substrate recognition in RNF8 may be further controlled by regions outside the RING domain.

The structural equivalent mutation in the Ring1B RING domain (K93D) inhibited the E3 ligase function of the RING domain dimer of Ring1B/Bmi1 toward nucleosomal H2A (Figure S3H), in accordance with recent data that identify this small loop as DNA binding region in Ring1B (Bentley et al., 2011). In contrast, we could not detect significant binding to DNA by the RING domain

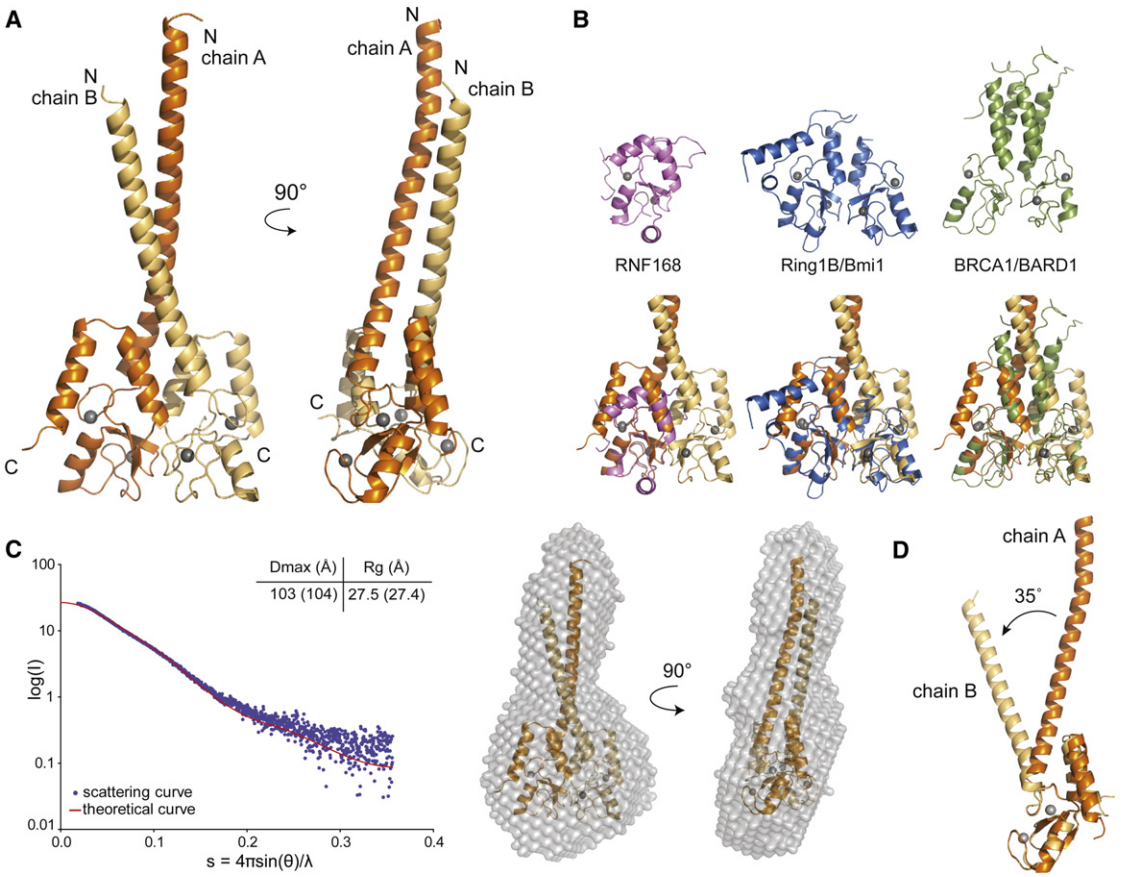

Figure 2. Structure of RNF8 RING Homodimer

(A) Cartoon representation of the crystal structure of the dimeric RING domain of RNF8 (residues 351–483 (chain A) and 359–485 (chain B) could be built in density, Zn^{2+} gray spheres). Front and 90° rotated view. N and C termini are highlighted.

(B) The RNF8 heterodimer resembles other RING E3 ligases: RNF168 monomer (magenta, PDB: 3L11), Ring1B/Bmi1 (blue, PDB: 2CKL), BRCA1/BARD1 (green, PDB: 1JM7). Superpositions with RNF8 shown without the extended N-terminal helices for clarity.

(C) SAXS analysis of the RNF8 RING domain. Plot of the scattering curve used for analysis and theoretical curve calculated from the crystal structure. Ab initio model (shown as light gray surface, derived from DAMAVER) based on SAXS measurements superposed to the crystal structure (orange cartoon). Table shows calculated values and expected ones are in brackets based on crystal structure.

(D) Superposition of chain A and B of the RNF8 RING dimer shows asymmetry due to a kink of the N-terminal flanking helices of 35°.

Images were prepared using GraphPad Prism and PyMOL. See also Figure S2 and Table S1.

of RNF168 (affinity weaker than 100 μM in 150 mM NaCl). Interestingly RNF168 R57D still modifies free H2A, but it clearly has reduced activity when other histone proteins are present (Figure S3J) indicating that nucleosomal proteins are also important for substrate recognition by this region in RNF168.

Apparently this site near the RING domain can either positively or negatively determine recognition of nucleosomal H2A by these E3 ligases and different aspects of the nucleosome contribute to this recognition process.

RNF168 Modifies Lys13 or 15 on H2A and H2AX In Vitro and In Vivo

Our findings suggest that the E3 initiating H2A/H2AX monoubiquitination during DSB signaling is RNF168. Because in vitro RNF168 and UbcH5c are able to attach more than one ubiquitin moiety to H2A/H2AX, we tested if these were chains using a ubiquitin variant without lysines (Ub K0). We still observe multiple bands, showing that RNF168 catalyzes multiple monoubiquitination of the histone, rather than forming chains, in these in vitro experiments (Figure 4A).

So far, the known site of monoubiquitination on H2A is K119 and in case of a mutation of this lysine, K118. These are targeted by the Polycomb PRC1 complex, where the Ring1B/Bmi1 dimer is the main E3 ligase (Wang et al., 2004). We wanted to investigate the site of ubiquitination of RNF168 in vitro and in vivo.

Using purified oligonucleosomes that contain mutated FLAG-tagged H2A variants, we show that RNF168 does not target the same site as Ring1B. In vitro we observe a preference for K13-15 (Figure 4B), as shown by its reduced ability to modify the K13-15R nucleosomal H2A variant. The RING domain construct of RNF168 is sufficient to provide specificity for these lysines

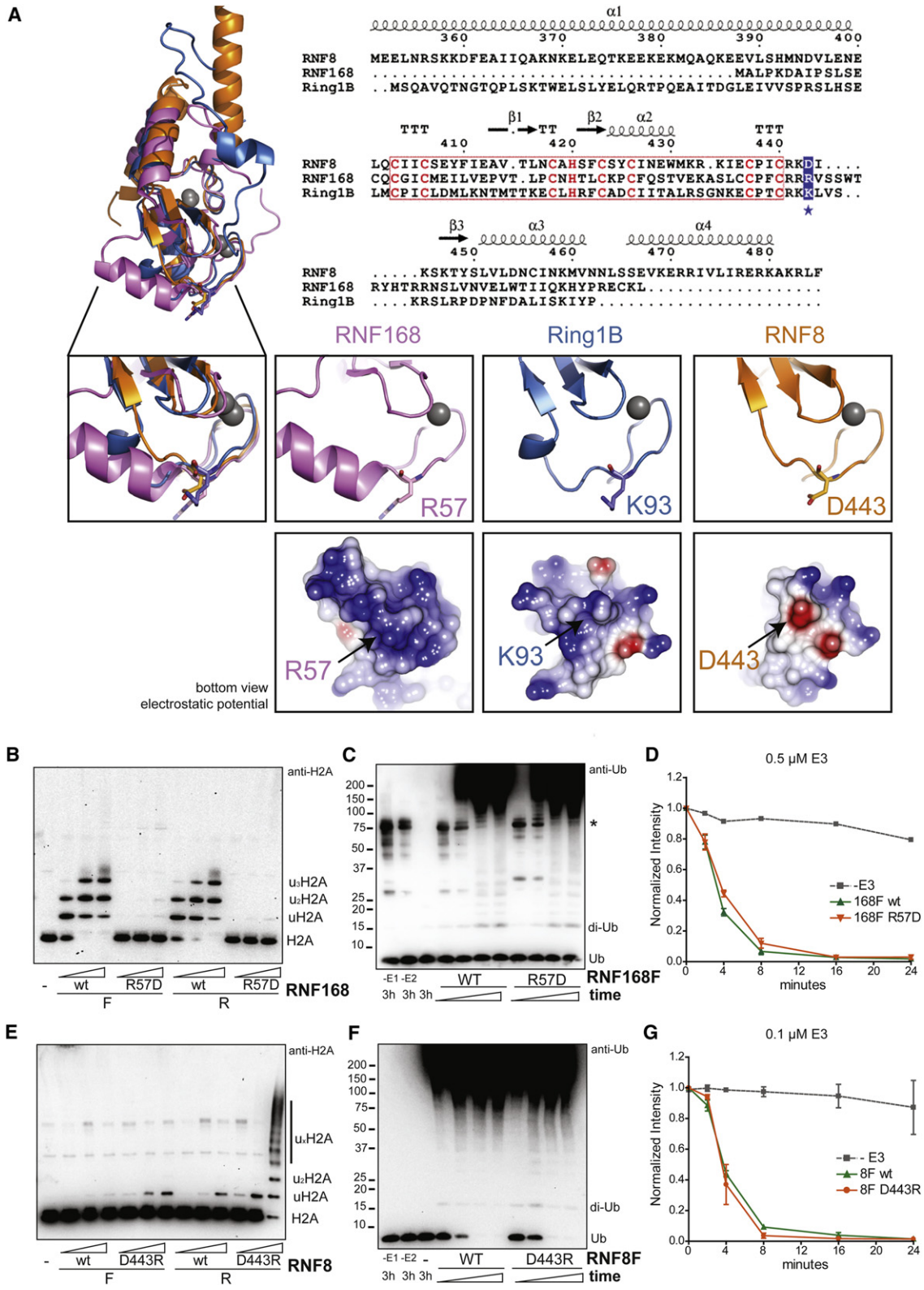

Figure 3. Determination of Substrate Recognition Site in E3 RING Domains

(A) Superposition of RNF8, Ring1B, and RNF168 RING domain structures (shown without RNF8 flanking helices) and structure based sequence alignment of the RING domains (with RNF8 secondary structure and numbering) reveals differences downstream of the core RING domain (red box highlights the core Zn-binding

(Figure S4B) and mutation of the ubiquitin binding domains MIU1 (A179G) (Doil et al., 2009; Pinato et al., 2009; Stewart et al., 2009) or UMI (L149A) (Pinato et al., 2011) on this construct do not affect its specificity (Figure S4B).

Interestingly the specificity for K13-15 of RNF168 is not present against the isolated H2A (Figure S4C). Like the specificity of RNF168 itself, as reflected by the R57D mutant (Figure S3J), the specificity for the H2A site seems to rely on higher order organization in the nucleosome.

In contrast to RNF168, Ring1B/Bmi1 has high specificity for K118-119, while the K13-15R mutation does not significantly affect its ability to target H2A in nucleosomes (Figure 4C).

K13-15 are located on the N-terminal tail of H2A whereas K118-119 are at the C terminus. As the H2A fold crosses the nucleosome, both tails are at the DNA interface, but diametrically opposite on the nucleosome rim (Figure S4A) (Luger et al., 1997). The K118-119 is located between the incoming and leaving strand of the DNA, whereas the K13-15 site is much more exposed. This site is close to the C-terminal tail of histone H2B (Figure S4A). Functionally one could therefore expect different downstream consequences to signals that arise from the RNF168- versus the Polycomb-dependent modification.

To validate our results in the cellular context, we used *H2ax*$^{-/-}$ mouse embryonic fibroblasts (MEFs) (Celeste et al., 2002). We reconstituted them with different mutants of H2AX (Figure S4D) and tested whether we could detect a modification of the histone at this site. We used the K118-119R H2AX mutant to remove the background Polycomb-dependent monoubiquitination. When we immunoprecipitated γH2AX after ionizing radiation (IR) treatment, we could observe a band corresponding to monoubiquitinated H2AX protein. This band was not present when K13-15 were also mutated, confirming that the modification takes place on these N-terminal residues (Figure 4D). This modification was induced by DNA damage as we could see that the ubiquitination at K13-15 was increased upon IR (Figures 4E and S4E).

Comparable results were obtained in human 293T cells where we introduced mutants of FLAG-H2A. Upon immunoprecipitation of the histone we could observe a damage-dependent ubiquitination, only when K13-15 were present (Figures 4F and S4E). This specific modification was fully abolished when RNF168 was depleted in these cells, confirming that this ligase is responsible for the monoubiquitination of H2A at K13-15 (Figures 4G and S4F).

Two major E3 ligases are also known to take part in DDR after DSBs: RNF8, that acts upstream of RNF168 allowing its recruitment (Doil et al., 2009; Pinato et al., 2009; Stewart et al., 2009) and BRCA1, that is thought to act downstream of RNF168 (Kim et al., 2007; Sobhian et al., 2007). When we depleted RNF8 in these experiments, we observed a overall decrease of the signal and the loss of IR-dependent induction, confirming the role of RNF8 in recruiting RNF168 to chromatin in DDR and suggesting that a background K13-15 modification is present in these cells due to RNF168 activity (Figures 4H, and 4I, and S4G). Depletion of BRCA1 did not significantly affect the K13-15 ubiquitination mark, supporting the fact that K13-15 modification is an early event in the DSB signaling (Figures 4H, 4I, S4F, and S4G).

K63-Linked Ubiquitin Chains on H2A/H2AX Are Formed on K13-15 In Vivo

Previous studies have proposed that K63 linked ubiquitin chains attached to H2A/H2AX and formed by RNF8 and RNF168 are the functional signal for the DSB repair pathway (Al-Hakim et al., 2010; Panier and Durocher, 2009).

To understand how these two ligases can achieve this modification on the histones, we studied a possible collaboration between these enzymes. As shown in Figure 4A, RNF168 can provide multiple monoubiquitination of H2A in nucleosomes incubated with the E2 UbcH5c. We observed that no additional activity was induced when RNF8 is added to this reaction (Figure 5A).

Because the E2 Ubc13 is important for the DSB pathway (Huen et al., 2007; Stewart et al., 2009; Zhao et al., 2007) and catalyzes K63 chains when complexed with the UEV cognate proteins (Hofmann and Pickart, 1999), we included this E2 in our assays. In presence of both E3s and of both E2s, UbcH5c and the complex Ubc13/Mms2, there was a significant increase of chains on H2A, that is dependent on both E2s and both E3 ligases (Figures 5A and S5A). These chains were not formed if Mms2 (UEV2) was omitted (Figure S5B).

To confirm that the RNF8 dependent ubiquitin chains on H2A are K63-linked, we performed a denaturing immunoprecipitation (IP) on nucleosomes containing FLAG-tagged H2A upon

RING domain, Zn coordinating residues in red). The residue that is positively charged in E3 ligases targeting H2A (Ring1B, RNF168) but negative in RNF8, that does not modify H2A in nucleosomes is shown (blue star in alignment, zoom and surface charge (blue positive, red negative), in boxes below).

(B) R57D mutation in full-length RNF168 causes loss-of-function toward H2A in nucleosomes (for antiubiquitin blot of these samples, Figure S3A).

(C) Full-length RNF168 R57D is not affected in ubiquitin chain formation capacity, time course experiment (10-30-90-180 min) performed with UbcH5c and in absence of oligonucleosomes (RING domain shown in Figure S3B).

(D) Single turnover E2 discharge is not affected by R57D mutation. Graphs showing UbcH5c discharge rates for full-length RNF168 WT and R57D (0.5 μM concentration E3). Mean and SD calculated on two independent experiments, and example SDS-PAGE used for analysis in Figure S3C.

(E) Gain-of-function D443R mutation in full-length RNF8 confers ability to ubiquitinate H2A with UbcH5c but to a lesser extent than the RING domain (for antiubiquitin blot of these samples, Figure S3D, time course assay Figure S3I).

(F) D443R mutation in full-length RNF8 does not affect ubiquitin chain forming capacity, time course experiment (10-30-90-180 min) performed with UbcH5c and in absence of oligonucleosomes (Figure S3E shows experiment for the RING domain).

(G) Single-turnover E2 discharge is not affected by D443R mutation. Graphs showing UbcH5c discharge rates for full-length RNF8 WT and D443R (0.1 μM concentration E3). Mean and SD calculated on two independent experiments, example SDS-PAGE used for analysis in Figure S3F. Assays shown in (C) and (F) are performed with 15 μM of ubiquitin. E3 concentration series in (B) and (E) (0.17-0.5-1.5 μM).

*Background bands. Alignment was done using ESPript. Images were prepared using GraphPad Prism, PyMOL, and CCP4mg (electrostatic potential). Ring (R), full-length (F). See also Figure S3.

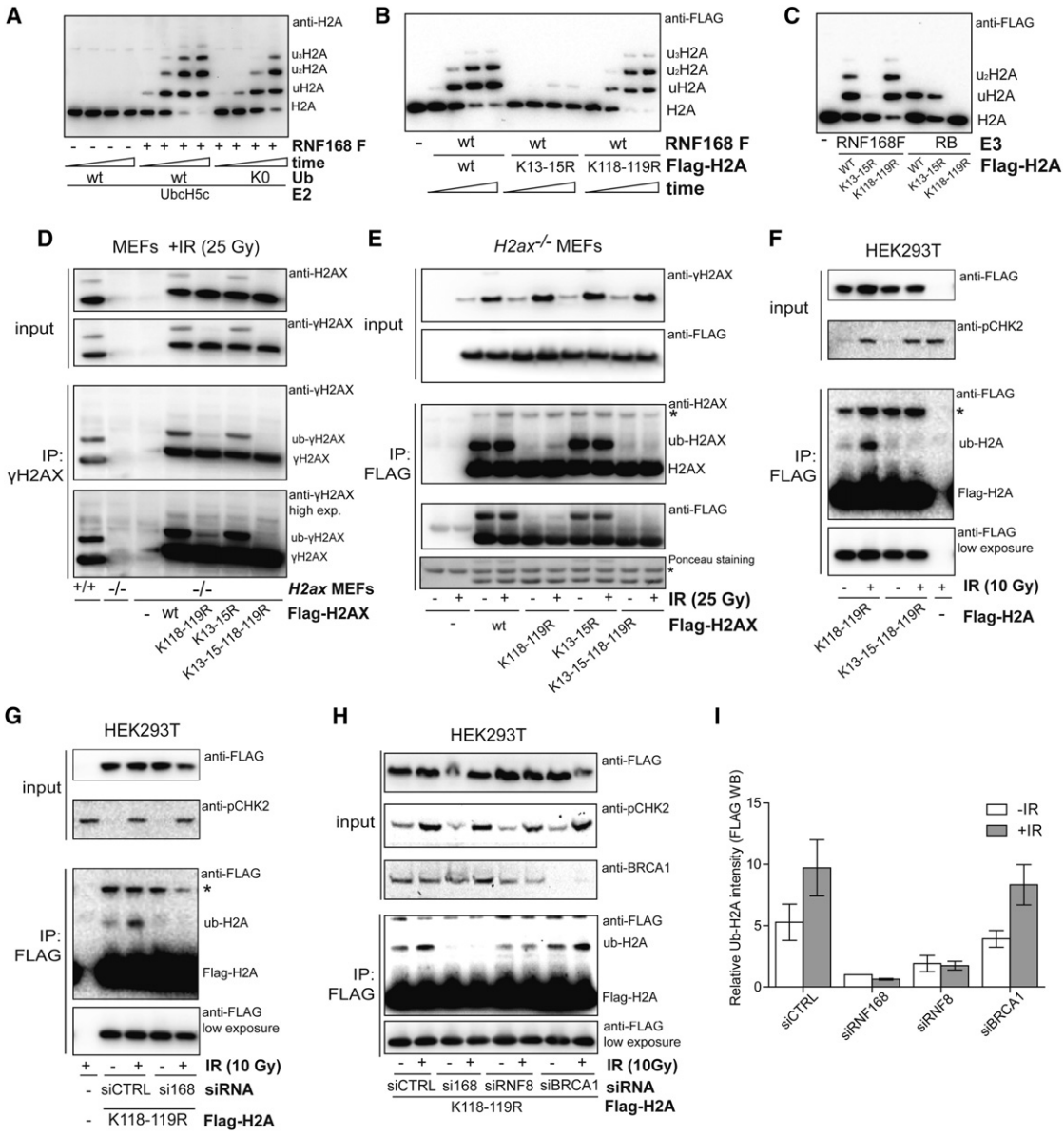

Figure 4. RNF168 Modifies K13-15 on H2A and H2AX In Vitro and In Vivo

(A) In vitro time course assay (10-30-90-180 min) to compare RNF168 activity with UbcH5c toward nucleosomal H2A with WT or K0 mutant ubiquitin. The Ub K0 reaction is slower because of surface changes.

(B) Time course assay (10-30-90-180 min) with oligonucleosomes containing different mutants of FLAG-tagged H2A.

(C) Full-length Ring1B/Bmi1 (RB) and RNF168 have different site specificity on H2A in nucleosomes. 2 hr assay performed with oligonucleosomes containing FLAG-tagged H2A constructs. Ubiquitin was used at 5 μM concentration in (B) and (C).

(D) γH2AX IP from WT MEFs or knockout for *H2ax* and reconstituted with different FLAG-H2AX constructs shows presence of a ubiquitinated form of H2AX only when K13-15 are present.

(E) FLAG IP from *H2ax*$^{-/-}$ MEFs reconstituted with different FLAG-H2AX constructs to test DNA-damage dependence of K13-15 ubiquitination.

(F) FLAG IP from HEK293T cells transiently transfected with different cDNA of FLAG-H2A mutants shows that ubiquitination on K13-15 is increased after IR.

(G and H) FLAG IP from HEK293T cells transiently transfected with different siRNA and cDNA of FLAG-H2A mutants shows that RNF168 is responsible for K13-15 modification. BRCA1 knockdown has no significant effect, while depletion of RNF8 affects the total level of the modification and its IR-dependence, confirming a role for RNF8 in recruiting RNF168. Control qRT-PCRs are shown in Figures S4F and S4G.

(I) Quantification of K13-15 H2A ubiquitination after treatment with different siRNAs. Data are shown relative to the siRNF168 condition in absence of IR. Error bars show SEM calculated from three independent experiments used for each condition. In anti-FLAG blots in(F)–(H), secondary anti-mouse antibodies specific for heavy chain were used.

*Background bands. See also Figure S4.

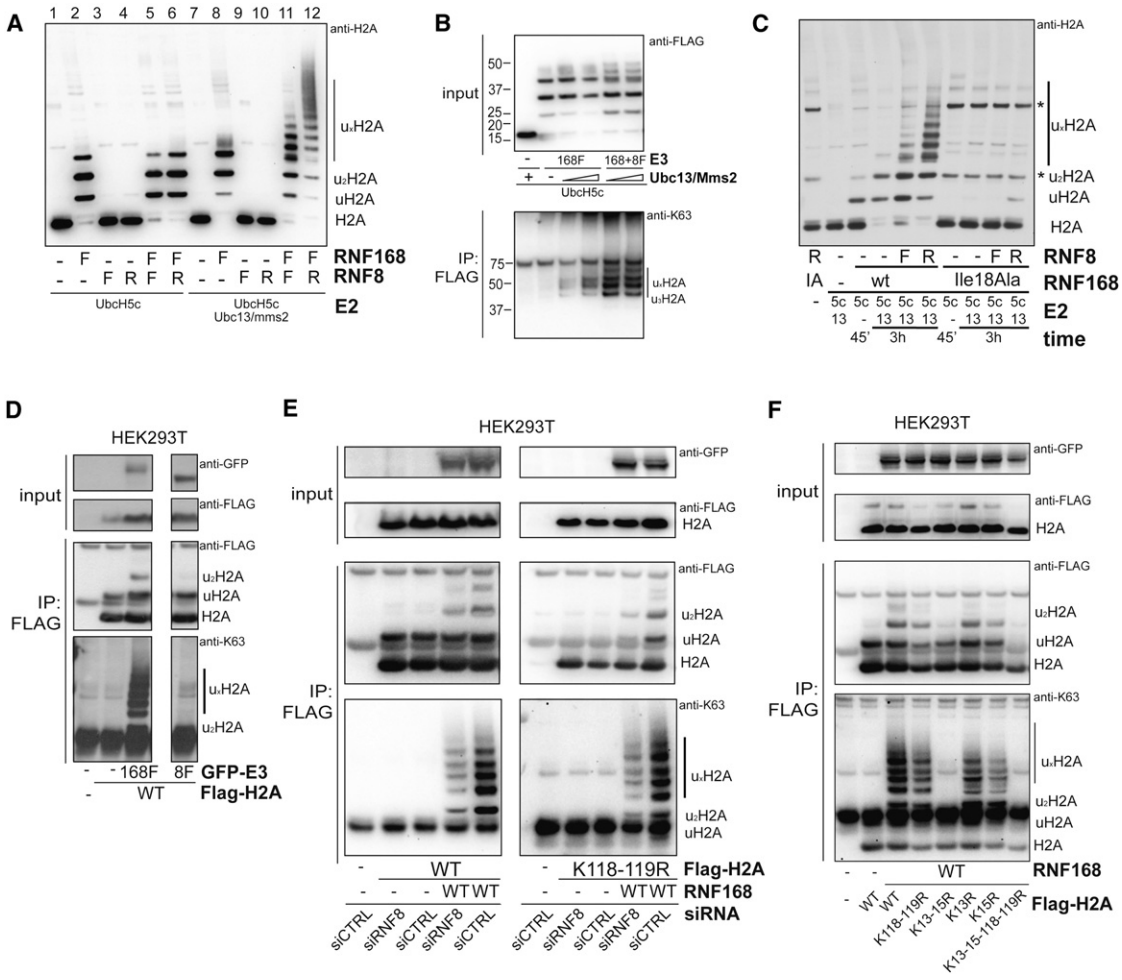

Figure 5. K63-Linked Ubiquitin Chains on H2A and H2AX Are Specifically Conjugated to K13-15

(A) When RNF8 is added to UbcH5c and RNF168, no additional activity is observed toward H2A (lane 5-6) over RNF168 activity. When Ubc13/Mms2 is added, RNF8 makes chains on ubiquitinated H2A (lane 11-12).

(B) Denaturing FLAG IP was performed after in vitro assay where nucleosomes contained FLAG-H2A. RNF8 efficiently forms K63 ubiquitin chains, while only mild activity is seen by RNF168. Ubc13/Mms2 was used in equimolar amounts compared to UbcH5c (0.5 μM) and at higher concentration (0.8 μM).

(C) Two-step assay, where nucleosomes were first incubated with RNF168 and UbcH5c to yield monoubiquitination and then Ubc13/Mms2 and RNF8 constructs were added to the samples to catalyze K63 chains. RNF8-dependent ubiquitin chain extension is dependent on the catalytic activity of RNF168. GST-tagged RNF168 wt and Ile18Ala (IA) were used. *Background bands.

(D) FLAG IP from HEK293T cells transiently transfected with GFP-RNF168 or GFP-RNF8 and FLAG-H2A. No additional ubiquitination of H2A was seen when RNF8 was overexpressed.

(E) Denaturing FLAG IP from HEK293T cells transiently transfected with GFP-RNF168, different siRNAs and FLAG-H2A (WT in left panel, K118-119R in right panel). K63 chains on H2A are dependent on RNF8. Control qRT-PCRs for RNF8 mRNA levels are shown in Figure S5H.

(F) K63 chains are specifically formed on K13-15 of H2A. Denaturing FLAG IP from HEK293T cells transiently transfected with GFP-RNF168 and different FLAG-H2A constructs.

K63 chains were visualized using the Genentech anti-K63 antibodies. See also Figure S5.

incubation with the ubiquitin machinery followed by immunoblotting with the specific anti-K63 antibodies (Newton et al., 2008) (Figure 5B). We observed only minor K63 modification if RNF8 is omitted from the reaction (Figure 5B). Similar results were obtained using a K63R mutant ubiquitin in the reaction (Figure S5C). These results confirm that, although a minor activity is retained by RNF168, K63 chains on histone H2A in nucleosomes are efficiently extended in vitro by RNF8 and Ubc13/Mms2, once the histone is monoubiquitinated.

Using a two-step assay, where we prime H2A for ubiquitination with RNF168 and UbcH5c and later add RNF8 and Ubc13/Mms2 we show that the two reactions are uncoupled. The priming activity of RNF168 and UbcH5c is required for chain formation, because the use of a ligase-inactive mutant (Brzovic et al., 2003) of RNF168 results in unmodified H2A (Figure 5C). Furthermore, the addition of RNF8 and Ubc13/Mms2 on other monoubiquitinated substrates (e.g., H2A ubiquitinated by Ring1B, PCNA (Zhang et al., 2008), or H2B) also leads to K63 chain formation on

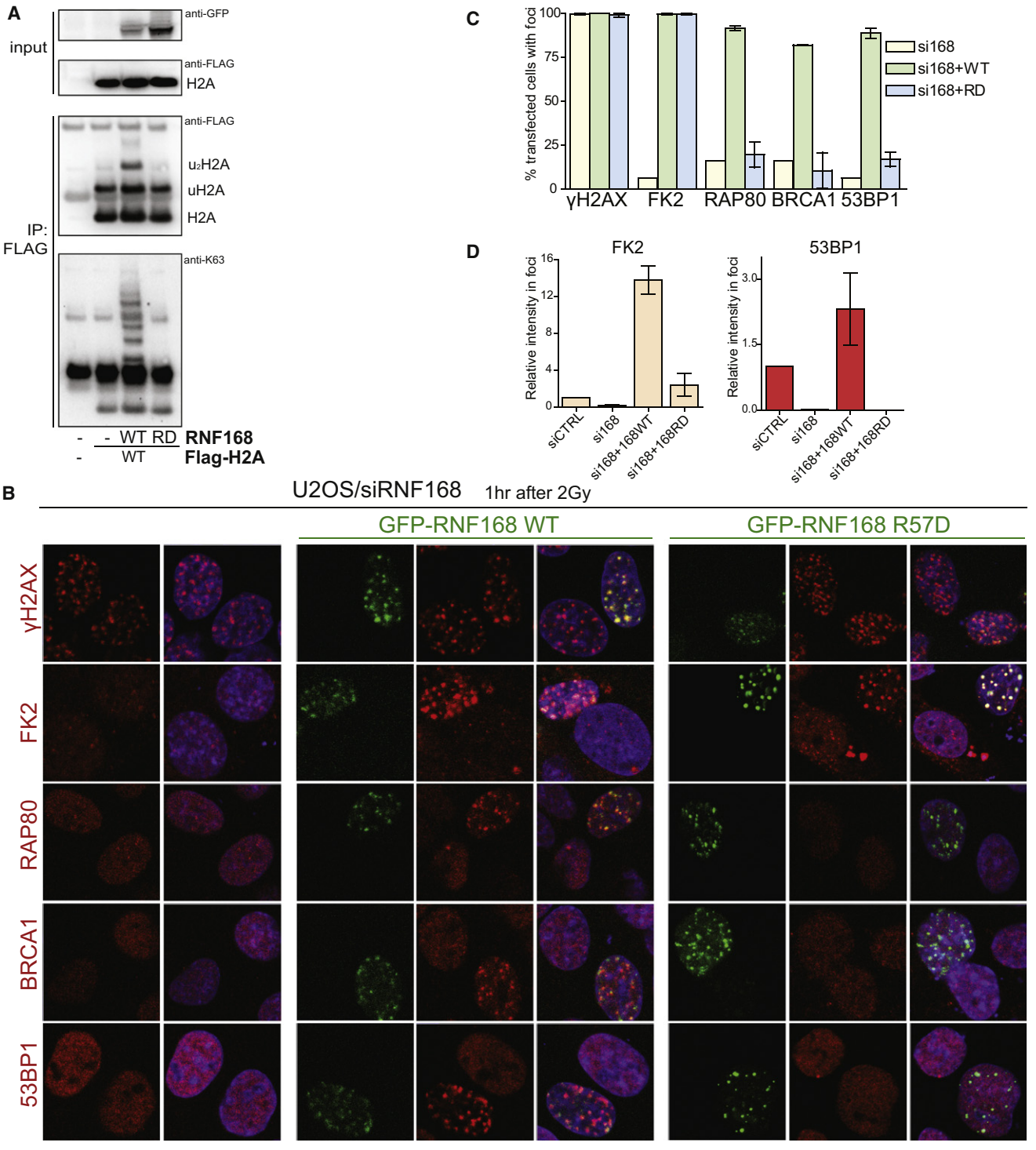

Figure 6. RNF168-Dependent Target Ubiquitination Is Required for Proper DSB Signaling

(A) Loss-of-function mutation R57D in RNF168 inhibits the ligase toward H2A in cells. Denaturing FLAG IP from HEK293T cells transiently transfected with GFP-RNF168 constructs and FLAG-H2A.

(B) Loss-of-function in RNF168 affects recruitment of RAP80, BRCA1, and 53BP1 to DNA damage foci. U2OS cells were transfected with siRNA against RNF168 and with GFP-tagged siRNA-resistant constructs of either empty vector, WT RNF168 or RNF168 R57D, treated with 2 Gy and stained for recruitment of indicated molecules 1 hr after irradiation. The R57D mutant rescues FK2 staining but not recruitment of downstream factors indicating that the H2A modification is critical for damage response.

these substrates (Figures S5D and S5F), suggesting that the Ubc13/RNF8 E2/E3 pair can efficiently extend ubiquitin chains on previously monoubiquitinated proteins.

The formation of K63 chains on H2A in vitro is efficiently achieved with a two-step reaction where two separate E2/E3 pairs are involved: RNF168 and UbcH5c are responsible for the priming of H2A, and RNF8 and Ubc13/Mms2 catalyze the chain extension of the monoubiquitinated protein. Such a two-step mechanism, where priming and chain extension are dependent on different ligases, provides tight regulation of target modification as shown for the polyubiquitination of the replication factor PCNA (Hoege et al., 2002).

We wanted to investigate K63-linked ubiquitin chain formation in cells during DSB signaling. We initially set out to monitor the chain formation on endogenous histones and therefore immunoprecipitated γH2AX after IR in different cell lines. However, we were never able to observe this type of chains (Figure S5G), suggesting that cellular levels of this modification are low or very transient.

To increase the level of monoubiquitinated H2A in vivo, we elevated the expression of RNF168. This resulted in robust K63 ubiquitin chains, specifically conjugated to H2A, as observed after denaturating IP of the histone in absence of IR (Figure 5D). Expression of RNF168 is sufficient to drive this polyubiquitination, while RNF8 does not induce this modification (Figure 5D). Nevertheless, this ubiquitination appears to be RNF8 dependent, as depletion of RNF8 in these cells causes a drastic reduction of this polyubiquitin chain consistent with our in vitro data (Figures 5E and S5H). Further unraveling of the relative contributions of RNF8 and RNF168 to H2A ubiquitination is complicated by the tight inter-relation between RNF8 and RNF168 functions during DDR, where activity of RNF168 is dependent on its recruitment by RNF8 and where RNF168-dependent H2A monoubiquitination is required for chain extension.

Interestingly, K63 polyubiquitin chains are not formed when K13-15 are mutated, whereas the mutation of K118-119 does not significantly affect ubiquitin chain formation on H2A (Figures 5E and 5F). Thus the K63 chain formation takes place on K13-15 and not on the Polycomb ubiquitination site in vivo.

This monoubiquitination by RNF168 in cells is specifically targeted to H2A-type histone, because no increase in H2B ubiquitination was observed upon nondenaturing IP of histone proteins, despite the proximity of the H2B tail to the K13-15 site (10–15 Å) (Figure S5I).

These results demonstrate that RNF168-dependent H2A monoubiquitination tightly directs the location of K63 ubiquitin chains during DSB repair and suggest a role for RNF8 in chain extension on this site on H2A.

Target Ubiquitination Is Required for Proper DSB Signaling

To test whether these findings concerning the specific site of ubiquitination and the order of events that leads to H2A/H2AX modification are relevant during DSB signaling we followed recruitment of factors such as 53BP1 and BRCA1 to DSBs upon DNA damage.

In *H2ax*$^{-/-}$ MEFs reconstituted with lysine mutant variants of H2AX, we were unable to observe any significant differences in the recruitment of 53BP1 and BRCA1 to the sites of damage (Figure S6A) indicating that the presence of endogenous wild-type (WT) H2A is sufficient to allow proper signaling. The large number of genes (~16) encoding for histone H2A precludes decreasing the levels of endogenous protein. Therefore we took advantage of the RNF168 target recognition site mutant (R57D) to address whether H2A/H2AX modification is an important step in the ubiquitin-dependent DSB signaling.

First, we tested whether this single point mutation also abolishes H2A/H2AX ubiquitination in cells. Therefore we expressed RNF168 and its loss-of-function mutant R57D in human 293T cells and monitored their ability to target the histone. Wild-type RNF168 strongly enhances H2A ubiquitination and allows K63 chain formation, while the R57D mutant is inactive toward H2A/H2AX (Figures 6A and S6B for H2AX), confirming that this charge reversal affects the target modification also in vivo.

To assess the integrity of the DSB signaling pathway in presence of this RNF168 mutant, we depleted U2OS cells of endogenous RNF168. As shown by others, this renders the cells unable to accumulate ubiquitin chains at the site of damage, which in turn impairs recruitment of RAP80, BRCA1, and 53BP1 (Figures 6B and 6C).

We then reconstituted these cells with siRNA-resistant constructs of either RNF168 WT or R57D mutant. As expected the WT protein fully restored the pathway, allowing accrual of downstream DSB factors (Figures 6B and 6C). In contrast the mutant RNF168 R57D was not able to drive the recruitment of RAP80, BRCA1, and 53BP1 (Figures 6B and 6C).

However, in contrast to RNF168 knockdown, complementation with this mutant resulted in significant formation of ubiquitin chains at the site of damage, as seen by FK2 staining (Figures 6B and 6C). This finding reflects the in vitro results, where the R57D mutation does not affect chain formation by RNF168. Notably, these ubiquitin chains stain positive for K63, but not K48 chains (Figures S6C and S6D), in line with published data showing that K48 chains occur at earlier time points after damage as a result of RNF8 activity and are independent of RNF168 (Feng and Chen, 2012; Meerang et al., 2011; Ramadan, 2012).

It is unclear what the docking site of these RNF168-dependent ubiquitin chains is, although autoubiquitination of the ligase could play a role. The total FK2 signal in foci per cell expressing RNF168 R57D mutant is lower than for cells complemented with WT protein (Figure 6D), consistent with the lack of chains on H2A/H2AX. Although this signal is comparable to the endogenous FK2 signal in control cells (siCTRL), the RNF168 R57D mutant does not rescue 53BP1 recruitment (Figure 6D) showing that ubiquitin chains themselves are not sufficient to signal, but

(C) Quantification of transfected cells with foci for the different markers shown in (B). Error bars show the SEM calculated from two independent experiments where 50 cells were counted per condition.

(D) Quantification of the total signal present in foci per cell for FK2 and 53BP1 based on two independent experiments similar to the ones shown in (B). Error bars show the SEM. At least 30 cells were counted per condition in each experiment.

See also Figure S6.

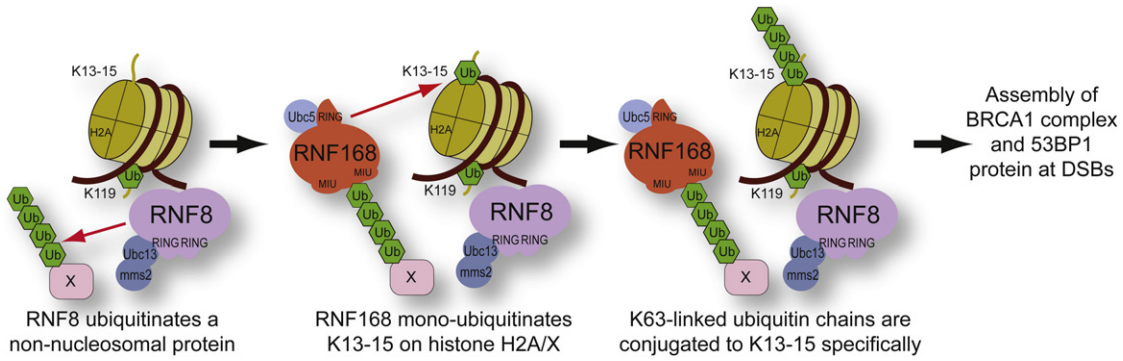

Figure 7. Proposed Model for RNF8- and RNF168-Dependent Activity at DSBs

RNF168 is recruited to DSBs by the activity of RNF8 toward a nonnucleosomal target. RNF168 monoubiquitinates H2A-type histones on K13-15 and this leads to K63 Ub chain formation on these lysines, which is required for proper DSB signaling.

that H2A/H2AX ubiquitination is the crucial signal that drives DSB signaling.

Overall these results show that proper modification of H2A is necessary for recruitment of downstream effector proteins in the DDR, while the presence of K63 ubiquitin chains per se is not sufficient for signaling.

DISCUSSION

Our study sheds light on the molecular details of the ubiquitination of H2A/H2AX during DSB signaling. Based on the order of their recruitment to the site of damage, RNF8 was previously described as the first ligase to target these histones for ubiquitination, while RNF168 was thought to be involved in the extension of such modification. We show that the previously described order of recruitment for these ligases does not predict the order in which they target H2A/H2AX (Figure 7).

We show that histone ubiquitination during the DSB pathway is initiated by RNF168 on H2A and H2AX, whereas RNF8 is inactive toward them (Figure 1). This finding is further explained by our structure-based mutagenesis, where we identify a single residue that is responsible for target recognition in the RING domain of these ligases (Figures 2 and 3). Mutation at this site affects H2A/H2AX modification but does not alter the ubiquitin chain forming capacity of these ligases, in vitro or in vivo (Figure 3).

We identify an H2A/H2AX ubiquitination site for the activity of RNF168, K13-15 (Figure 4). We show that during DDR, K63 chains are formed specifically on this site, which distinguishes the DNA damage induced modification from Polycomb-mediated K119 monoubiquitination (Figure 5). Importantly, we show that histone modification at the damage site is required for proper DSB signaling, because the mere formation of ubiquitin chains is not sufficient to drive the response pathway (Figure 6).

Revised Model for RNF8 Function in H2A/H2AX Ubiquitination during DSB Signaling

Our findings challenge the current model for H2A/H2AX ubiquitination during DSB signaling. Once RNF8 accumulates at the lesions, its catalytic activity is required to form ubiquitin chains that will recruit RNF168 (Doil et al., 2009; Pinato et al., 2009; Stewart et al., 2009). In this study, we provide evidence that RNF168 is the priming ligase for histones; therefore, the chains that are responsible for the recruitment of RNF168 must be conjugated to a different substrate (Figure 7). RNF8 was recently suggested to be involved in the ubiquitination of other proteins localized at the site of damage (Acs et al., 2011; Feng and Chen, 2012; Mallette et al., 2012; Meerang et al., 2011), These and possibly more yet to be discovered ubiquitination products of RNF8 might be the docking site for the recruitment of RNF168. Additionally, we show that RNF8 is highly active in making ubiquitin chains (Figure 1B), suggesting that RNF8 could catalyze free chains in proximity of the site of damage similarly to what happens with K63 ubiquitin chains during the NF-kB signaling (Xia et al., 2009).

Once RNF168 is recruited to the DSB, it will initiate H2A/H2AX ubiquitination on K13-15, where K63 chains will then be extended (Figure 7). We show that in vitro RNF8 efficiently catalyzes formation of this type of chains on the ubiquitinated histones, suggesting that collaboration between the two ligases can take place on H2A/H2AX, with RNF168 catalyzing the priming reaction and RNF8 efficiently extending the K63 chains.

Distinct Monoubiquitination Sites on H2A/H2AX May Signal Differently

Our study shows that RNF168 is responsible for a ubiquitination on histone H2A/H2AX on K13-15, a different site than the known Polycomb site K118-119. The nucleosomal H2A targeting site on the E3 ligases does not account for this H2A lysine specificity, since Ring1B and RNF168 both have a positive charged targeting site, but differ in lysine choice.

We provide evidence that K63 chains are specifically formed on the DDR dependent K13-15 ubiquitination site, suggesting that during DSB signaling the priming reaction of RNF168 is important to tightly control the pathway and to maintain distinct ubiquitin signals on the same target, H2A/H2AX.

Recent studies show involvement of the Polycomb E3 ligase proteins Ring1B/Bmi1 in the DSB repair pathway (Chagraoui et al., 2011; Chou et al., 2010; Facchino et al., 2010; Ginjala et al., 2011; Ismail et al., 2010; Pan et al., 2011; Wu et al.,

2011). Apparently both modifications on H2A are important during the DDR, but since they are located on opposite sides of the nucleosome (Figure S4A) they could provide independent signals in the DDR. On the one hand, monoubiquitination of K119 could be important for the transcriptional silencing of the regions around the damage, as previously suggested (Chagraoui et al., 2011; Shanbhag et al., 2010). On the other hand, the K13-15 polyubiquitination could represent a signal that not only allows the recruitment of downstream proteins of the DSB cascade through the K63 chain (e.g., RAP80 binding), but also might induce nucleosomal rearrangements that are important during the assembly of the repair machinery through the actual location of this ubiquitination. In fact, K13-15 of H2A are located in proximity to K120 of H2B, which is also a target for monoubiquitination in late DSB signaling and in actively transcribed regions where it favors the opening of chromatin (Moyal et al., 2011; Nakamura et al., 2011; Shiloh et al., 2011). This could suggest that RNF168-dependent ubiquitination at K13-15 might induce similar open conformations of the chromatin around the site of damage. It is an interesting question whether the site of attachment of these ubiquitin chains might act as a more complex regulator of the chromatin organization around the damage rather than merely a recruitment station.

EXPERIMENTAL PROCEDURES

Details of experiments are presented in the Extended Experimental Procedures.

Cell Culture and Generation of DSBs

MEFs were received from A. Nussenzweig. All cell lines were cultured in DMEM containing 10% fetal bovine serum. *H2ax*$^{-/-}$ and wild-type MEFs were cultured at 3% oxygen concentration. Retroviruses carrying FLAG-tagged H2AX constructs were used to generate reconstituted stable cell lines. IR was delivered using a ^{137}Cs irradiation unit with a dose rate of 1 Gy/min.

Protein Preparations and Crystallography

Full-length RNF8 and Ring1B/Bmi1 were expressed in insect cells, RNF168 and the RING domain constructs were made in *Escherichia coli*. Oligonucleosomes were purified from human cells as described (Buchwald et al., 2006). Crystals were grown of the RNF8 RING domain (351–485). Crystallographic data collection and refinement statistics are shown in Table S1.

Small Angle X-Ray Scattering

Samples for the small angle x-ray scattering (SAXS) experiments were prepared in five different concentrations (from 0.4 to 7 mg/ml) in gel-filtration buffer. Data were collected at EMBL Hamburg (beamline ×33). Data were analyzed using the ATSAS software package (Svergun et al., 2001).

In Vitro Ubiquitination Assays

Purified human Uba1 at 0.2–0.6 μM was mixed with E2s (0.5 μM), the E3 ligases (0.5–2 μM), ubiquitin (100 μM in all assays unless otherwise stated), ATP (3 mM), and 10 μM of H2A in oligonucleosomes. The reactions were incubated at 32°C for 3 hr (unless otherwise stated) in buffer 50 mM Tris/HCl (pH 7.5), 100 mM NaCl, 10 mM $MgCl_2$, 1 μM $ZnCl_2$, 1 mM TCEP. Specificity for K13-15 on H2A for RNF168 is achieved by incubation with lower excess of free ubiquitin (below 10 μM).

E2 Discharge Assays

UbcH5c was loaded with ubiquitin in presence of E1 and Mg^{2+} and ATP. After stopping the reaction with EDTA, this mixture was incubated with the E3s or buffer as control. Samples were analyzed by SDS-PAGE and quantified.

Immunoprecipitations

For FLAG IPs, transiently transfected 293T or stable MEF cell lines were harvested directly after irradiation. After a PBS wash, cells were lysed and sonicated in E1A buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 0.1% Tween-20) in presence of phosphatase, protease inhibitors. In denaturing IPs, 0.5% SDS was added to the lysates after sonication. The samples were then diluted ten times and incubated with beads to avoid denaturation of the antibodies.

For γH2AX IPs, cells were irradiated and were directly harvested. Acidic extraction of histone proteins was performed from isolated nuclei. After neutralization, histones were incubated O/N with anti-γH2AX antibodies (Millipore) and pulled down with protein G beads (GE Healthcare).

Immunofluorescence Studies

U2OS cells were first transfected with siRNAs, followed by DNA transfections 24 hr later. Cells were damaged with ionizing radiation (2 Gy) 48 hr after siRNA transfection and fixed in 2% paraformaldehyde in presence of 0.1% Triton X-100 1 hr after damage. Coverslips were washed and incubated at room temperature with primary antibody for 1 hr. After incubation for 1 hr with secondary antibody, samples were embedded in DAPI containing Vectashield mounting medium (Vector). Quantifications were done using confocal images, analyzed by the Fiji Software.

ACCESSION NUMBERS

Coordinate and structure factor were deposited in the Protein Data Bank under identification code 4AYC.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, six figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2012.08.005.

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