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# Differential regulation of RNF8-mediated Lys48- and Lys63-based poly-ubiquitylation

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## ABSTRACT

Pairing of a given E3 ubiquitin ligase with different E2s allows synthesis of ubiquitin conjugates of different topologies. While this phenomenon contributes to functional diversity, it remains largely unknown how a single E3 ubiquitin ligase recognizes multiple E2s, and whether identical structural requirements determine their respective interactions. The E3 ubiquitin ligase RNF8 that plays a critically important role in transducing DNA damage signals, interacts with E2s UBCH8 and UBC13, and catalyzes both K48- and K63-linked ubiquitin chains. Interestingly, we report here that a single-point mutation (I405A) on the RNF8 polypeptide uncouples its ability in catalyzing K48- and K63-linked ubiquitin chain formation. Accordingly, while RNF8 interacted with E2s UBCH8 and UBC13, its I405A mutation selectively disrupted its functional interaction with UBCH8, and impaired K48-based poly-ubiquitylation reactions. In contrast, RNF8 I405A preserved its interaction with UBC13, synthesized K63-linked ubiquitin chains, and assembled BRCA1 and 53BP1 at sites of DNA breaks. Together, our data suggest that RNF8 regulates K48- and K63-linked poly-ubiquitylation via differential RING-dependent interactions with its E2s UBCH8 and UBC13, respectively.

## INTRODUCTION

Ubiquitylation shares three common enzymatic steps orchestrated by the concerted actions of ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2) and ubiquitin ligase (E3) (1,2). Like many other

post-translational protein modification systems, ubiquitin conjugates serve as molecular switches to regulate and fine-tune processes that include protein stability and protein–protein interactions. Accordingly, the complexity of protein ubiquitylation, illustrated by diverse linkage patterns and length of poly-ubiquitin chains, determines the nature and the functional consequences of these conjugation events.

Ubiquitin is a 76 amino acid polypeptide that harbors seven lysine (K) residues (K6, K11, K27, K29, K33, K48 and K63). Mono-ubiquitylation involves formation of an isopeptide bond between a single ubiquitin moiety and a lysine residue of its target proteins. Via one of the seven lysine residues on ubiquitin, ubiquitin chains on protein conjugates can be extended, giving rise to diverse ubiquitin chain topologies (3,4). Moreover, recent studies also uncovered formation of linear ubiquitin chains that involves linkages between N- and C-terminal of ubiquitin (5). While functions of many of these distinct ubiquitin chains remain obscure, poly-ubiquitin chains composed of K48-linkages are generally associated with commitment for proteasomal degradation, whereas K63-linked poly-ubiquitylation plays established roles in DNA damage–repair, protein kinase activation and receptor endocytosis (6–8).

The Ring Finger Protein RNF8 is an ubiquitin ligase that belongs to the RING-type subfamily. The RNF8 polypeptide harbors two conserved domains, namely the phospho-peptide-binding FHA (Forkhead-Associated) and the E3 ubiquitin ligase signature RING (Really Interesting New Gene) motif. While the RNF8 FHA mediates its interaction with the DNA damage mediator protein MDC1 and allows its relocalization to DNA damage sites, its C-terminal RING domain has been shown to recruit the E2 ubiquitin-conjugating enzyme UBC13 to facilitate the transfer of K63-linked poly-ubiquitin chains onto H2A-type histones

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surrounding DNA double-strand breaks (DSBs) (9–11). Thus, RNF8 contributes to the ubiquitin landscape at the damage-modified chromatin to allow productive and local accumulation of tumor suppressor proteins BRCA1 and 53BP1 (9–12). While the RNF8–UBC13 pair is pivotal in DNA damage signal transduction, RNF8 has also been reported to interact with other E2s, including UBCH8 (13,14). However, exactly how the RNF8–UBCH8 interaction is regulated and whether this interaction contributes to DNA damage–repair and/or other cellular processes remains elusive.

In this study, we describe a point mutation (I405A) on the RNF8 RING domain that uncoupled its K63- and K48-linked ubiquitylating activities. We found that RNF8 I405A interacted with UBC13 but not UBCH8, and was selectively compromised in promoting K48-based ubiquitin linkages.

## MATERIALS AND METHODS

### Cell cultures and transfection

The 293T and RNF8-deficient MEF cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum at 37°C in 5% CO<sub>2</sub>. Culture medium for MEF cells stably expressing various mutants of epiptope-tagged RNF8 was supplemented with 2 µg/ml puromycin. Cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

### Antibodies

Antibodies against γH2AX, 53BP1, BRCA1, ub-H2A and RAD18 were previously described (10). Conjugated ubiquitin was detected by anti-FK2 (Upstate Cell Signaling). Anti-Flag (M2) and anti-actin antibodies were from Sigma. Anti-myc (9E10) and anti-HA antibodies were from Covance.

### Expression constructs

cDNAs-encoding RNF8, UBC13, MMS2, UBCH8 and ubiquitin were subcloned into pDONR201 using Gateway Technology (Invitrogen), and were subsequently recombined into Gateway compatible destination vectors according to manufacturer's protocol. Site-directed mutagenesis was performed to generate either deletion or point mutants of RNF8, UBC13, UBCH8 and ubiquitin, and desired mutations were confirmed by DNA sequencing.

### Co-immunoprecipitation and immunoblotting

Cells were harvested and lysed with NETN buffer (20 mM Tris–HCl, pH 8, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) 24 h post-transfection. Protein extracts were cleared by centrifugation at 13 000 rpm for 10 min at 4°C. A total of 10% of extract was used to detect the expression of transfected proteins while the remaining lysates were incubated with streptavidin-conjugated beads for 4 h at 4°C with gentle agitation. Beads were washed twice and boiled in SDS sample buffer for 10 min. Eluted proteins were then fractionated by

SDS–PAGE and electroblotted to Hybond-P membranes followed by incubation in 5% skim milk for 30 min. Membranes were incubated with primary antibodies at 4°C overnight and subsequently incubated with HRP-conjugated secondary antibodies (Jackson Immuno-research Laboratories) for 2 h. Signals on the membrane were visualized by enhanced chemiluminescence (Thermo Fisher Scientific).

### In vivo ubiquitylation assay

Cells were transfected with 2 µg flag-tagged RNF8 and myc-ubiquitin expression constructs. Twenty-four hours post-transfection, cells were incubated with 10 µM MG132 for 4 h prior to protein extraction. Immunoprecipitation of ectopic RNF8 was carried out under denaturing condition for 4 h at 4°C. Beads were boiled in SDS sample buffer and the eluted proteins were then analyzed by western blotting.

### In vitro ubiquitylation assay

Assay was performed with 0.1 µg E1 (Boston Biochem), 0.2 µg of purified UBCH8 or UBC13/Mms2, 1 µg of purified RNF8 WT, ΔRING and I405A and 5 µg of ubiquitin (Boston Biochem) in reaction buffer containing 50 mM Tris–HCl (pH 7.5), 10 mM MgCl<sub>2</sub>, 10 mM dithiothreitol and 1 mM ATP. Reaction was carried out at 37°C for 1 h and was stopped by boiling with Laemmli buffer.

### Cycloheximide treatment

To determine the stability of various forms of RNF8, transfected cells were incubated with 50 µg/ml of cycloheximide. Cells were harvested and lysates were separated by SDS–PAGE to analyze protein expressions at indicated time points.

### Immunofluorescence staining

Cells grown as monolayer on coverslips were exposed to ionizing radiation. After a 4-h recovery, cells were washed with PBS and fixed with 3% paraformaldehyde for 15 min at room temperature. Subsequently, cells were permeabilized in 0.5% triton solution for 3 min at room temperature. To detect ubH2A foci, cells were pre-extracted with triton solution followed by fixation with paraformaldehyde. Foci were stained by sequential incubations of primary antibodies and secondary fluorophore-conjugated antibodies for 1 h, respectively. Nuclei were visualized by staining with DAPI. Finally, coverslips were mounted and IR-induced foci were visualized using an Olympus BX51 fluorescence microscope.

### IR sensitivity assay

For the colorimetric proliferation assay, 800 cells grown on 96-well plate were irradiated with 0, 1, 2 and 5 Gy. Cells were allowed to grow for 5 days at 37°C. Cell viability was measured by Cell Proliferation Kit (XTT) (Roche) according to the manufacturer's manual.

Three independent experiments were performed each in triplicates.

For the clonogenic survival assay, 4000 cells were grown on 10 cm<sup>2</sup> plates and irradiated with 0 or 5 Gy. The number of colonies was determined by staining with Coomassie blue after growing for 10 days. Three independent experiments were performed each in triplicates.

## RESULTS

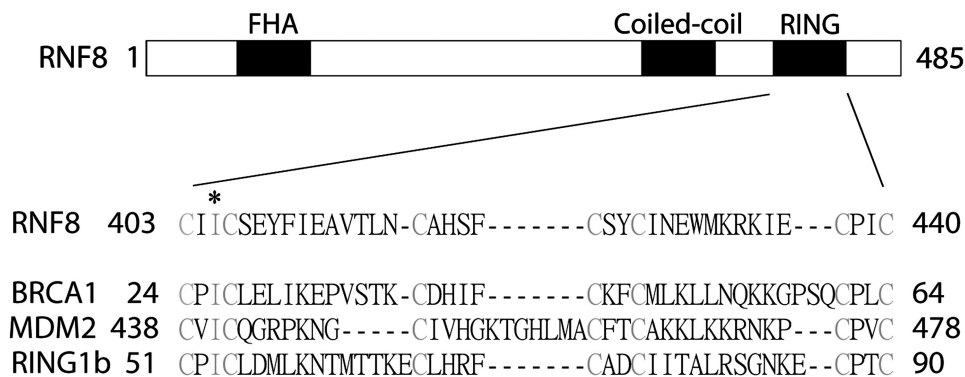
### Generating the RNF8 I405A mutation

The pairing of an E3 ubiquitin ligase with its E2 ubiquitin-conjugating enzymes allows productive ubiquitylation, and dictates synthesis of specific ubiquitin chain linkages. Indeed, while the DNA damage-responsive E3 ubiquitin ligase RNF8 interacts with E2s UBC13 and UBCH8, previous studies suggested that RNF8, via UBC13, synthesizes K63-linked ubiquitin chains at sites of DNA breaks and is critically important for proper cellular responses to DNA damage. While it is not entirely clear how RNF8 selects UBC13 to effect local ubiquitylation reactions, HERC2 was found to selectively promote RNF8–UBC13 but not RNF8–UBCH8 complex formation (13), suggesting possible structural determinants in these HERC2-regulated events. To examine this idea, we compared the amino acid sequence of RNF8 RING with RING domains derived from BRCA1, MDM2 and RING1b. Sequence alignment revealed a number of conserved residues across the different RINGs. One of these included the RNF8 cysteine residue (C403), which when mutated, has previously been shown to perturb the RING structure and compromise RNF8 ubiquitin ligase activity (14,15). In addition, we also identified a conserved hydrophobic isoleucine residue (I405) which resided within the first zinc-chelating motif (Figure 1). Accordingly, mutating this residue, as in the case of BRCA1, MDM2 and RING1b, abolished their interactions with their respective E2s without affecting the structure of RING domains (16–19). Thus, we hypothesized that mutation analogous to this isoleucine within the RNF8 RING domain may compromise its binding to its E2s and inactivate its E3 ligase activities.

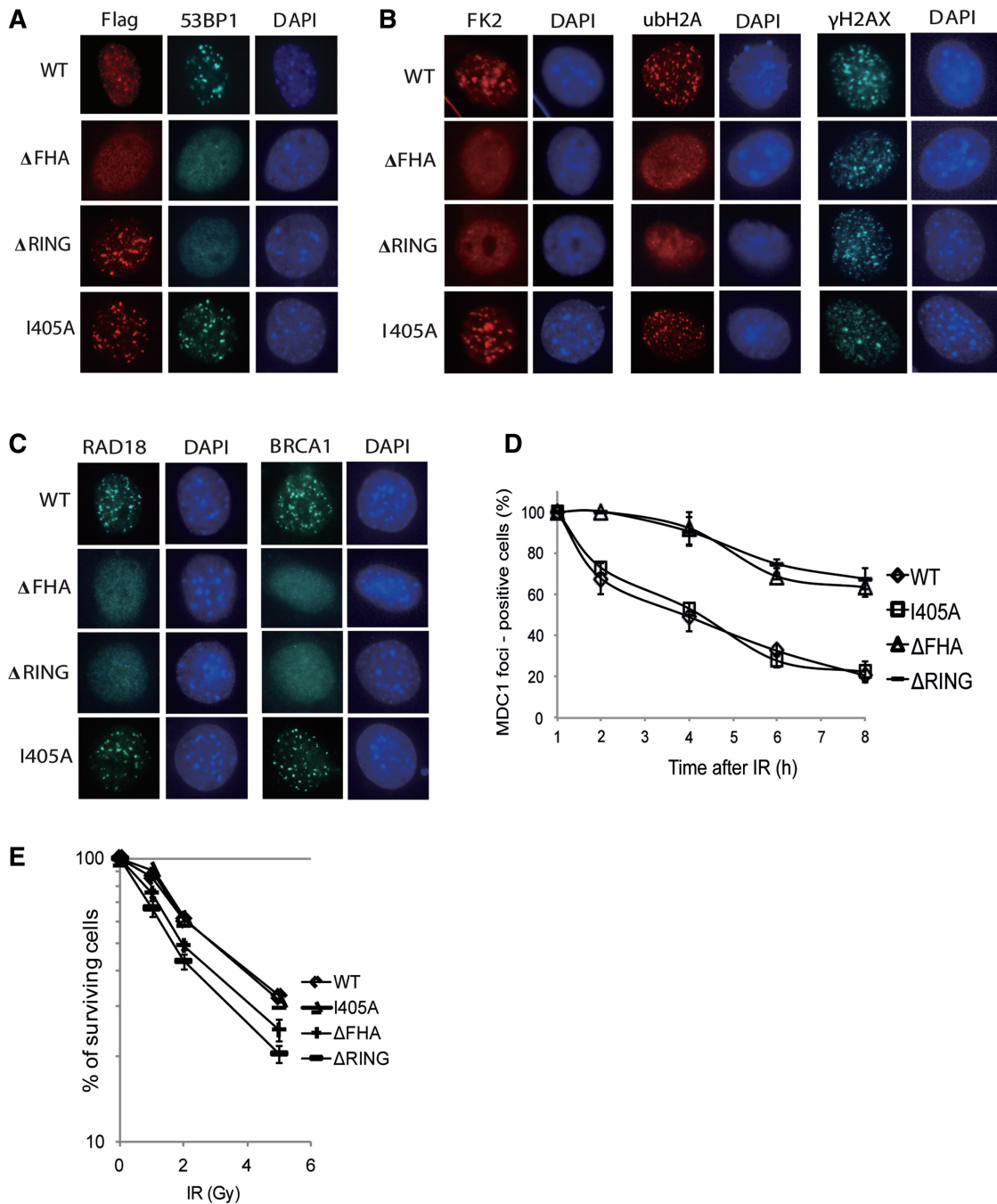
### RNF8<sup>I405A</sup> cells promotes DNA damage signaling

We generated an RNF8 mutant in which the isoleucine at position 405 was replaced by alanine (I405A). We assessed and compared this point mutant with a RNF8 RING domain deletion mutant in promoting DNA damage signals since RNF8 plays an established role in mediating DNA damage-induced histone ubiquitylation and in assembling tumor suppressors BRCA1 and 53BP1 at sites of DNA breaks (9–12). To this end, we retrovirally infected RNF8-deficient MEFs with expression cassettes encoding wild-type (WT) RNF8 or its various mutants ( $\Delta$ FHA,  $\Delta$ RING and I405A; Supplementary Figure S1). Consistent with the fact that the RNF8 is targeted to ionizing radiation-induced foci (IRIF) through its phospho-peptide-binding FHA domain-dependent interaction with MDC1 (9–11), we found that WT RNF8 and its RING mutants (I405A and  $\Delta$ RING), but not the RNF8  $\Delta$ FHA, localized to IRIF (Figure 2A). Moreover, while reconstitution of WT RNF8 functionally restored IR-induced 53BP1 foci,  $\Delta$ RING- and  $\Delta$ FHA-reconstitutions did not (Figure 2A). Interestingly, to our surprise, cells reconstituted with the RNF8 I405A mutant supported IR-induced focal accumulation of 53BP1 (Figure 2A), suggesting that I405A does not impair RNF8 functions in propagating DNA damage ubiquitin signals.

RNF8 promotes local ubiquitylation events at sites of DNA breaks (20). To further examine whether the RNF8 I405A mutant is functional in DNA damage signal transduction, we examined DSB-associated ubiquitin conjugates employing antibodies that stain conjugated ubiquitins (FK2) and ubiquitylated H2A molecules (ub-H2A). Similar to WT RNF8-expressing cells, but not those reconstituted with the  $\Delta$ FHA and  $\Delta$ RING mutants, RNF8 I405A promoted damage-induced ub-H2A and FK2 foci formation (Figure 2B). Additionally, we observed similar numbers of FK2 and 53BP1 IRIFs in RNF8-deficient MEFs expressing either WT RNF8 or its I405A mutant over a time course of 8 h (Supplementary Figure S2), suggesting that the RING domain point mutation (i.e. I405A) does not compromise the RNF8-mediated IR-induced ubiquitylation events at DNA damage sites.



**Figure 1.** Generating the RNF8 I405A mutation. Sequence alignment of RING domains of RNF8, BRCA1, MDM2 and RING1b was shown. Conserved cysteine and isoleucine (asterisk) residues were highlighted in grey.



**Figure 2.** RNF8<sup>I405A</sup> has an intact DDR pathway. (A–C) immunofluorescence staining of Flag epitope-tagged RNF8, 53BP1, FK2, ub-H2A, RAD18, BRCA1 and γH2AX foci were shown after 4-h recovery from 10 Gy IR. (D) RNF8 deficient MEFs reconstituted with RNF8 WT, ΔFHA, ΔRING and I405A were immunostained with MDC1 antibody at the indicated time points after exposure to 2 Gy IR. Cell with MDC1 foci exceeded 10 per nucleus was counted as positive and 100 cells were scored for each time point. (E) Radiosensitivity of RNF8 deficient MEFs reconstituted with WT, ΔRING and I405A of RNF8 was examined 5 days after exposure with various dose of IR by XTT. Error bars represent standard deviations of the mean from three independent experiments.

To extend these observations, we further analyzed the subcellular localization of BRCA1 and RAD18. Both of these E3 ubiquitin ligases have previously been shown to concentrate at DNA breaks in RNF8-dependent manners (9–12,21). In line with the possibility that RNF8 I405A functionally replaced WT RNF8 in DNA damage signaling, both RNF8 and its I405A mutant, but not ΔFHA

and ΔRING, promoted BRCA1 and RAD18 accumulation at IRIF (Figure 2C).

To further examine the ability of WT RNF8 and I405A in DNA damage–repair, we quantified MDC1 foci formation at different time points after challenging these cells with a recoverable dose of IR (2 Gy). MDC1 operates upstream of the RNF8-dependent ubiquitylation cascade,

and its focal accumulation is reflective of the cellular status of DNA damage. Accordingly, while we observed sustained numbers of MDC1 IRIFs in  $\Delta$ RING and  $\Delta$ FHA-expressing cells, MDC1 foci gradually resolved in RNF8-deficiency cells expressing either the WT or I405A RNF8 (Figure 2D), indicating that these RNF8 alleles exhibited similar DNA repair dynamics. Together with the fact that RNF8 I405A conferred cellular resistance to IR to similar extents to its WT counterpart in RNF8-deficient cells (Figure 2E and Supplementary Figure S3), we speculated that RNF8 I405A is an active ubiquitin ligase that is fully functional in promoting DNA damage-induced ubiquitin signaling.

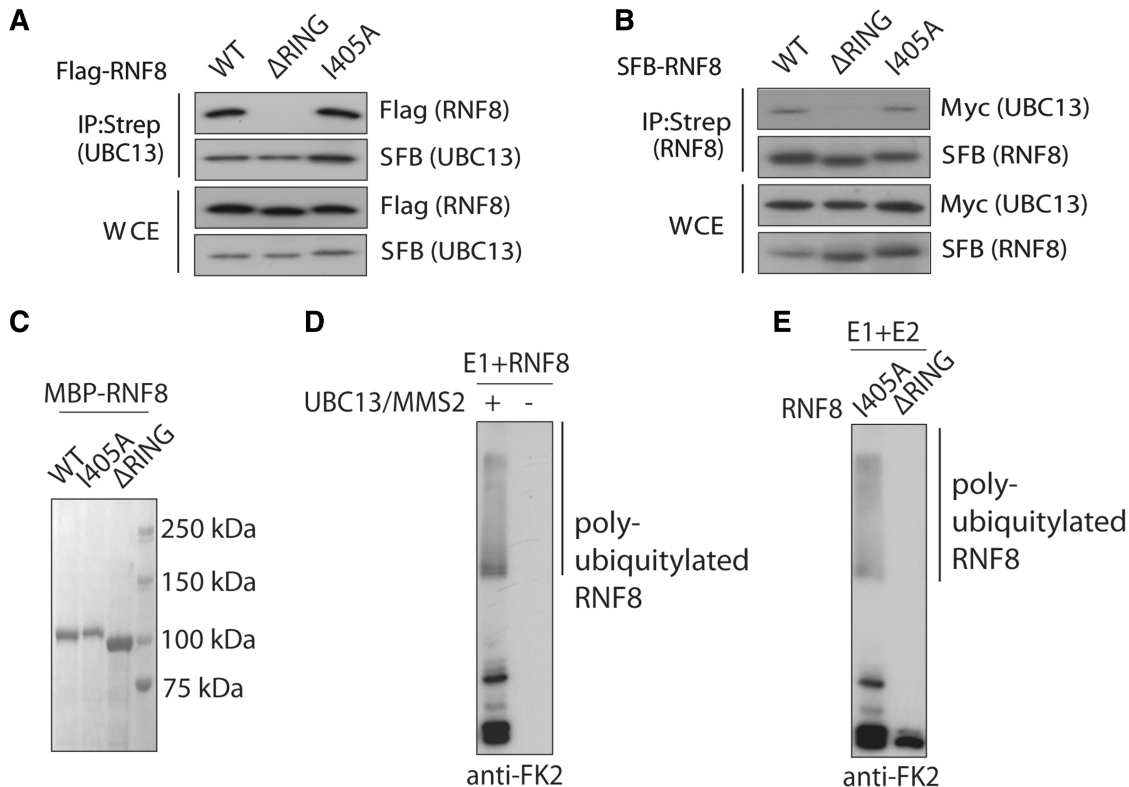
### RNF8<sup>I405A</sup> interacts with UBC13 to effect ubiquitylation

Previous studies indicated that RNF8 forms a complex with the E2 ubiquitin conjugating enzyme UBC13 to promote local histone ubiquitylation at the damaged chromatin, which in turn is critically important for recruitment and accumulation of a number of checkpoint and repair proteins (10,12,22). To evaluate if the I405A mutant interacts with UBC13, we performed co-immunoprecipitation experiments following co-transfection of UBC13 and

RNF8 or its mutants into 293T cells. Reciprocal binding assays clearly showed that, while the RNF8  $\Delta$ RING failed to interact with UBC13, both WT RNF8 and I405A physically associated with UBC13 (Figure 3A and B). Having shown that RNF8 I405A interacts with UBC13, we next assessed whether I405A is functionally active as an E3 ubiquitin ligase. Using *in vitro* ubiquitylation assays, we found that bacterially purified RNF8 proteins (Figure 3C) catalyzed ubiquitylation in the presence of UBC13/MMS2 (Figure 3D). Consistent with the fact that RNF8 I405A fully restored RNF8 deficiency in DNA damage signal transduction, we found that RNF8 I405A, but not  $\Delta$ RING, supported auto-ubiquitylation *in vitro* (Figure 3E). We concluded that the I405A mutation does not abrogate RNF8–UBC13 functions in transducing the ubiquitin-mediated DNA damage signals.

### Role of proteasome in regulating RNF8 protein stability

Thus far, our data indicated that RNF8 I405A interacted with UBC13, and functionally substituted WT RNF8 in promoting local histone ubiquitylation and assembly of 53BP1, RAD18 and BRCA1 at sites of DNA breaks.



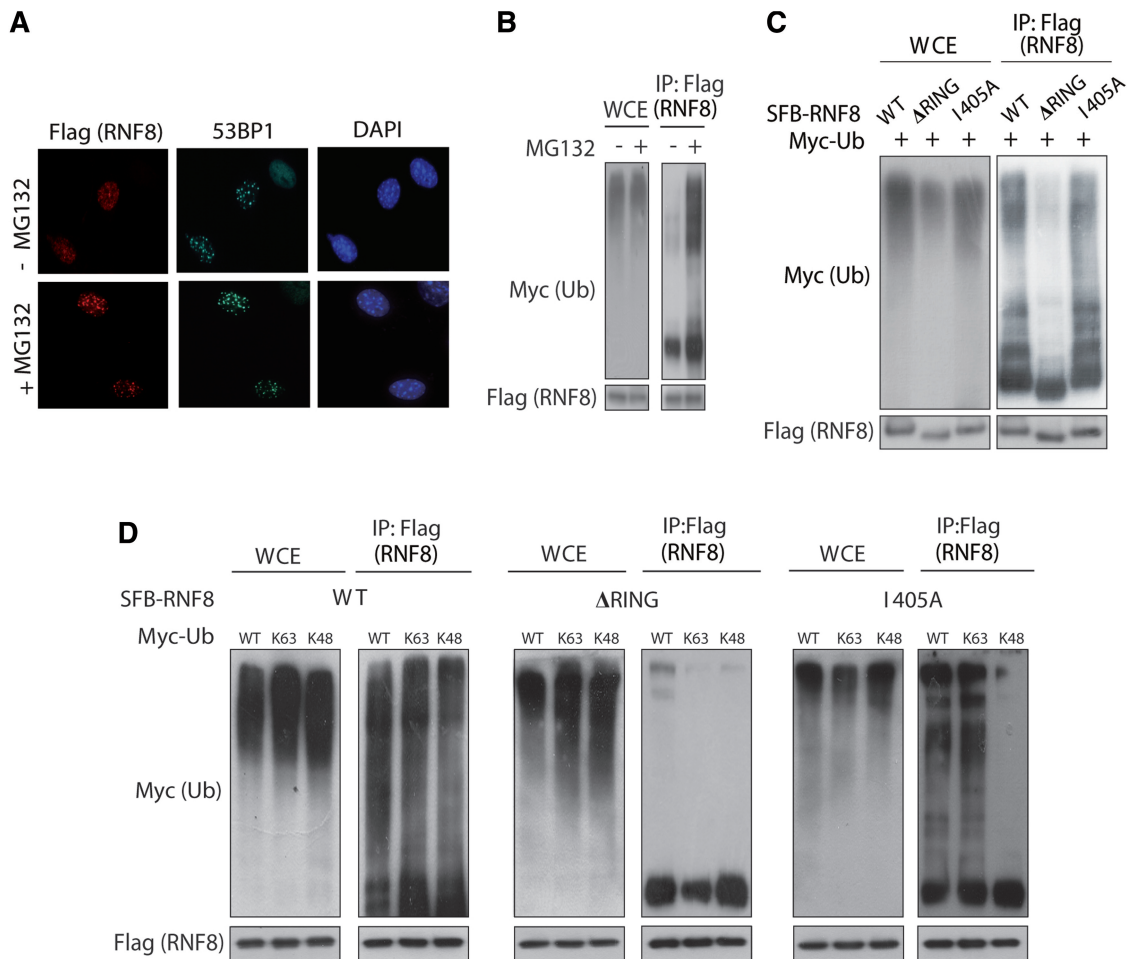
**Figure 3.** RNF8<sup>I405A</sup> interacts with UBC13 to effect ubiquitylation. (A) Association between RNF8 and UBC13 was assessed by immunoblotting RNF8 immunoprecipitates. 293T cells were co-transfected with plasmids-encoding Streptavidin-binding peptide Flag-tagged RNF8 (SFB-RNF8) or its mutants and myc-UBC13. Protein lysates were incubated with streptavidin-conjugated beads for 4 h at 4°C. Proteins were then eluted from beads, separated by SDS-PAGE and analyzed by western blot with indicated antibodies. Whole-cell extract (WCE) represents 10% of total proteins. (B) 293T cells were co-transfected with plasmids of SFB-UBC13 and flag-RNF8 or its mutants. UBC13 was immunoprecipitated and subjected to immunoblotting to detect its interaction with RNF8 and its mutants as described in (A). (C) Purified proteins of WT,  $\Delta$ RING and I405A of RNF8 were subjected to SDS/PAGE and visualized by staining with Coomassie blue solution. (D) *In vitro* ubiquitylation reactions were performed in the presence of WT RNF8 with or without E2 UBC13/MMS2. (E) *In vitro* ubiquitylation reactions were performed with  $\Delta$ RING or I405A of RNF8. Ability of RNF8 or its mutants to form polyubiquitin chain was examined by immunoblotting with FK2 antibody.

However, similar to  $\Delta$ RING, we observed that I405A IRIFs were much more readily detected than those of WT RNF8 (Figure 2A). This prompted us to ask if the RNF8 proteins may be actively and negatively regulated by the proteasome. To test this hypothesis, we inhibited the proteasome using MG132 and analyzed RNF8 IRIFs. Pre-treatment of MG132 was previously shown to inhibit 53BP1 IRIF by depleting the free ubiquitin pools in nucleus (11,23). To avoid this indirect effect of proteasome inhibition, we treated cells with low doses of MG132 only after IR challenge. Accordingly, addition of MG132 significantly increased the number and intensity of WT RNF8 foci (Figure 4A), suggesting that WT RNF8 proteins may be further stabilized at DNA breaks upon proteasome inhibition, and that RNF8 proteins may be regulated by proteasomal degradation. Importantly, 53BP1 IRIF was readily observed in the same MG132-treated cells, suggesting that the increased WT RNF8

foci formation is, to a large extent, due to inhibition of proteasomal degradation of RNF8 (Figure 4A).

**Mutation at I405A impairs K48 poly-ubiquitylation and stabilizes RNF8**

Our observation that RNF8 is negatively regulated by the proteasome suggested that the E3 ubiquitin ligase may be subjected to K48-linked poly-ubiquitylation *in vivo*. Indeed, we found that ubiquitylated species of RNF8 can be readily detected in the presence of MG132 (Figure 4B) (14). Moreover, these autoubiquitylation events were largely dependent on the RNF8 RING, as ubiquitylated species of RNF8  $\Delta$ RING were substantially reduced (Figure 4C). Consistent with the fact that RNF8 I405A interacted with UBC13 and promoted ubiquitin conjugations at sites of DNA breaks, we found that RNF8 I405A was proficient in catalyzing ubiquitin chain



**Figure 4.** Role of proteasome in regulating RNF8 protein stability. (A) Immunofluorescence staining of Flag epitope-tagged RNF8 and 53BP1 foci were shown 6 h following irradiation of 10 Gy either in the presence or absence of 1  $\mu$ M MG132. (B) 293 T cells were co-transfected with SFB-RNF8 and myc-ubiquitin (Myc-Ub). Twenty-four hours post-transfection, cells were incubated with or without 10  $\mu$ M MG132 for 4 h prior to protein extraction. Lysates were immunoprecipitated for 4 h at 4°C and eluted with SDS sample buffer. The ubiquitylation status of RNF8 was analyzed by immunoblotting with indicated antibodies. (C) 293T cells were co-transfected with SFB-RNF8 or its mutants and myc-ubiquitin. Cells were treated with MG132 for 4 h prior to protein extraction. Ubiquitylation of RNF8 and its mutants was assessed by immunoprecipitation and immunoblotting as described in (B). (D) Synthesis of ubiquitin chains by RNF8 and its mutants was assessed. 293T cells were co-transfected with SFB-RNF8 or its mutants and WT ubiquitin or ubiquitin K48- and K63-only mutants.

formation in the substrate-independent ubiquitylation assay (Figure 4C).

Prompted by the considerable difference of IRIF between WT RNF8 and I405A mutant, we speculated that RNF8 I405A might differ from RNF8 in its ability in catalyzing K48-linked ubiquitin chains. To test this, we transfected 293T cells with expression constructs of RNF8 or its mutants, together with either wild-type ubiquitin or its single lysine-containing mutants (i.e. K48 or K63 only). In contrast to RNF8  $\Delta$ RING, WT RNF8 became heavily modified not only by wild-type ubiquitin, but also by ubiquitin chains composed primarily of K48- or K63-ubiquitin linkages (Figure 4D). Consistent with our observation that RNF8 I405A preserved its interaction with the K63-ubiquitin chain promoting E2 UBC13, RNF8 I405A was post-translationally modified by K63-ubiquitin chains. Surprisingly, RNF8 I405A was specifically compromised in promoting K48-based ubiquitin chains (Figure 4D).

To further substantiate the notion that the RNF8 I405A mutation may selectively disrupt the RNF8-dependent K48-ubiquitin chain promoting activity, we performed co-immunoprecipitation assays to examine the binding of various forms of RNF8 with its cognate E2 UBCH8, which is responsible for K48-ubiquitin chains formation and has been associated with proteasomal degradation (14,24). Indeed, results obtained from reciprocal binding assays demonstrated that only WT RNF8, but neither

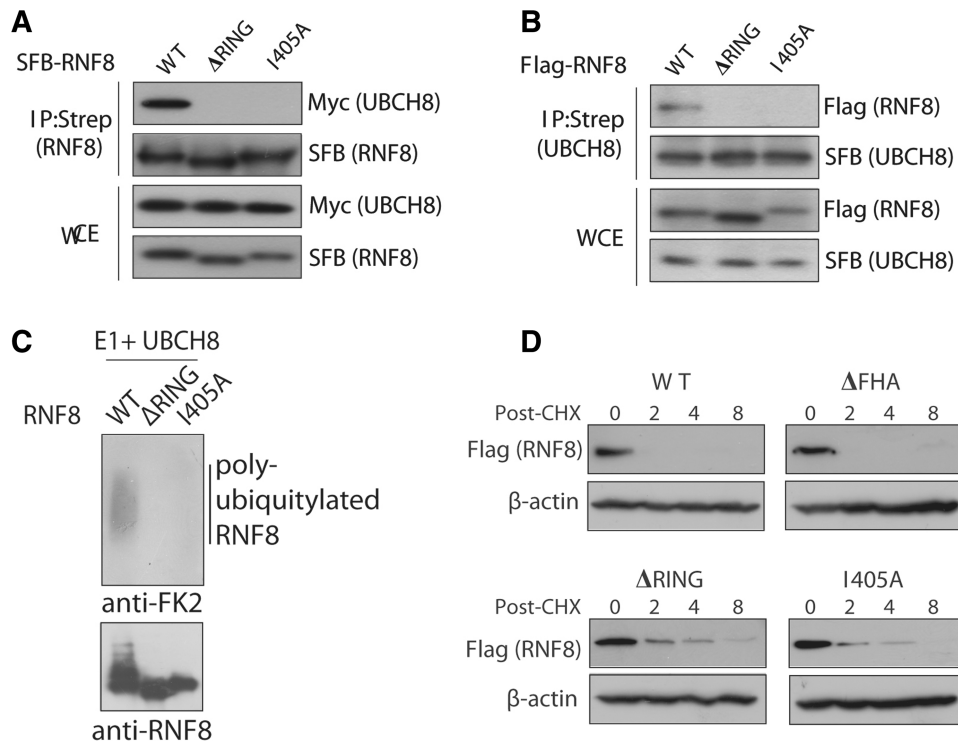
$\Delta$ RING nor I405A, associated with UBCH8 (Figure 5A and B). Consistently, WT RNF8, but not  $\Delta$ RING and I405A, promoted UBCH8-mediated ubiquitylation *in vitro* (Figure 5C). Together, our results support the idea that RNF8 interacts with UBCH8 and promotes K48-based poly-ubiquitylation, which is impaired in both the RNF8  $\Delta$ RING and I405A mutants.

Based on the observation that the RNF8 I405A mutation inhibited K48-ubiquitin chain formation, we asked if the RNF8 I405A mutation may promote protein stability. Upon cycloheximide treatment, we found that expression of WT RNF8 and its  $\Delta$ FHA mutant declined rapidly (Figure 5D), whereas the RNF8  $\Delta$ RING and I405A proteins exhibited enhanced stabilities.

### Roles of UBCH8 and UBC13 in RNF8-dependent ubiquitin conjugation

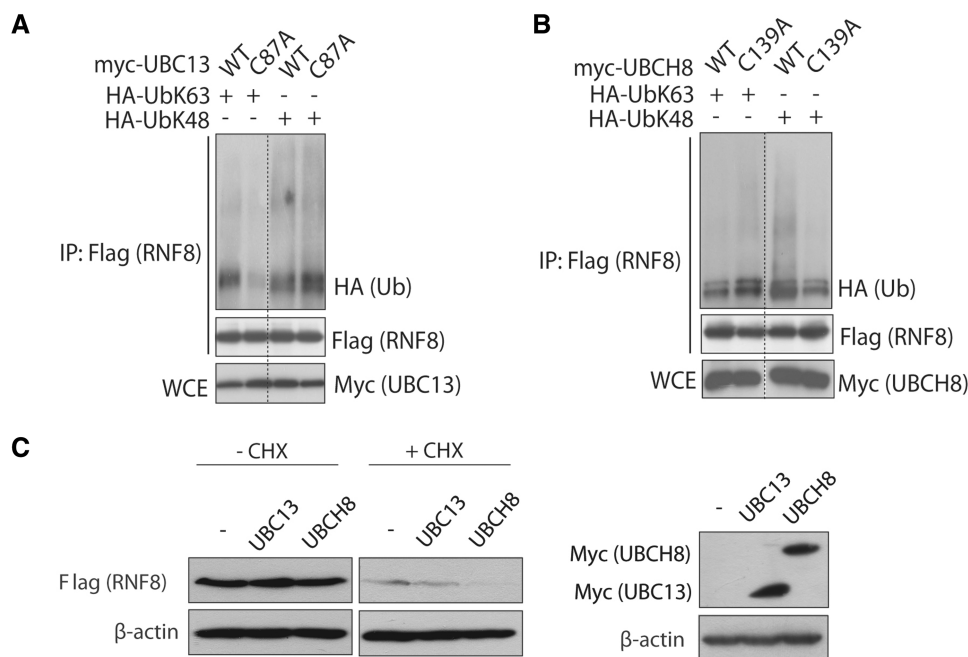
Next, we assessed the requirement for UBC13 and UBCH8 in promoting the RNF8-mediated K63- and K48-based ubiquitylation *in vivo*. We first examined roles of UBC13 and UBCH8 in catalyzing RNF8 autoubiquitylation. Consistently, we found that wild-type UBC13, but not its catalytically inactive C87A mutant, mediated K63-based poly-ubiquitylation (Figure 6A). Conversely, UBCH8, but not its C139A mutant, promoted ubiquitin chains composed primarily of K48-linkages (Figure 6B).

Given the possibility that the RNF8-UBCH8 interaction may regulate RNF8 protein turnover via



**Figure 5.** The RNF8 I405A mutation impaired its interaction with UBCH8. (A) 293T cells were co-transfected with plasmids encoding SFB-RNF8 or its mutants and myc-UBCH8. (B) 293T cells were co-transfected with SFB-UBCH8 and flag-RNF8 or its mutants. Interaction was detected by co-immunoprecipitation and immunoblotting. (C) *In vitro* ubiquitylation assay was performed with WT,  $\Delta$ RING and I405A of RNF8 in the presence of purified UBCH8 proteins. Reaction mixtures were incubated at 37°C for 3h, separated by SDS-PAGE and immunoblotted with indicated antibodies. (D) 293T cells were transfected with flag-RNF8 and its mutants. Cells were collected at the indicated time points following the addition of cycloheximide and protein lysates were subjected to immunoblotting to assess expression levels of RNF8 proteins.





**Figure 6.** Roles of UBC13 and UBCH8 in RNF8-mediated poly-ubiquitylation. (A) 293T cells were transfected with SFB-RNF8, WT UBC13 or its mutant (C87A) and ubiquitin mutants (K48 or K63). Cells were treated with MG132 for 4 h before protein extraction. Protein lysates were subjected to immunoprecipitation for 4 h at 4°C. Ubiquitylation status of RNF8 was determined by immunoblotting with indicated antibodies. (B) 293T cells were transfected with SFB-RNF8, WT UBCH8 or its mutant (C139A) and ubiquitin mutants (K48 or K63). Ubiquitylation of RNF8 was determined as described in (A). (C) 293T cells were co-transfected with flag-RNF8 and myc-UBCH8 or myc-UBC13. Cells were incubated with or without cycloheximide and expression of RNF8 was analyzed by immunoblotting (left). Ectopic expression of myc-UBCH8 and -UBC13 were shown by immunoblotting (right).

K48-based poly-ubiquitin chains, we further assessed RNF8 stability in the presence of overexpressed UBCH8. Accordingly, using UBC13 as control, we found that UBCH8 expression downregulated RNF8 protein levels (Figure 6C). Conversely, UBCH8 depletion stabilized RNF8 proteins in HeLa cells (Supplementary Figure S4A and S4B). Together, these data support our observation that the RNF8–UBCH8 module is important in catalyzing ubiquitin chains composed of K48 chains.

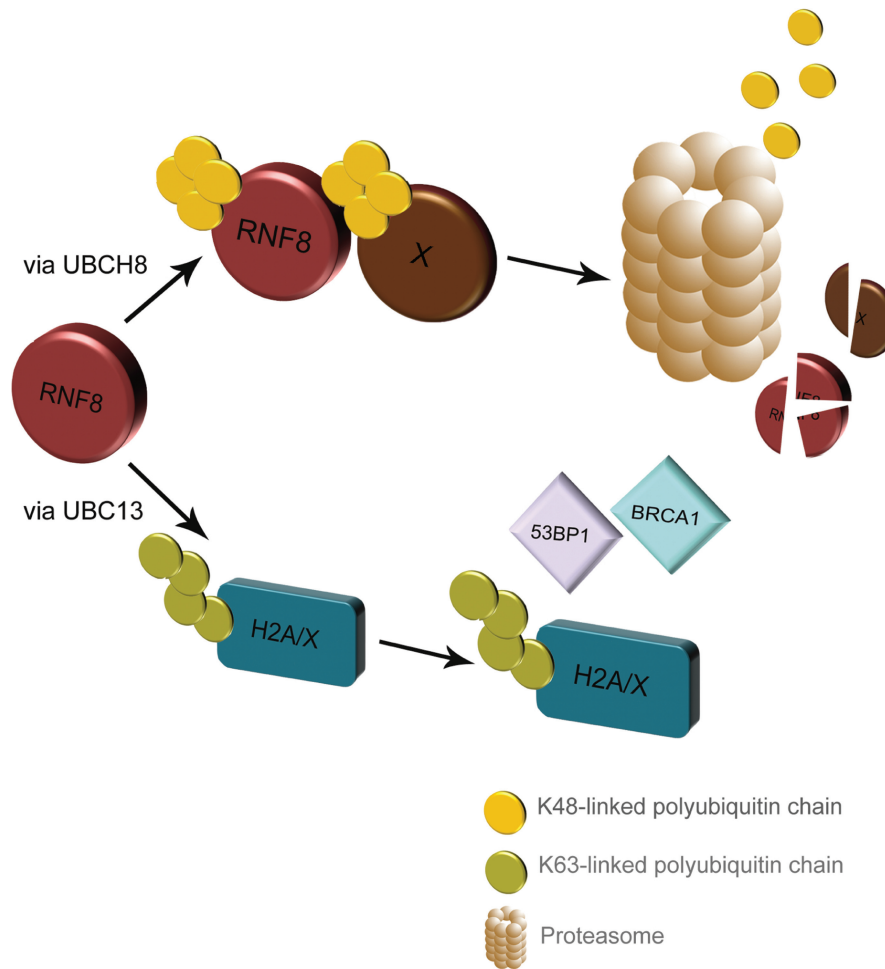
While RNF8 I405A interacted with UBC13, promoted DNA break-associated ubiquitylation, and assembled DNA repair proteins at sites of DNA breaks, this RING domain point mutation (I405A) abrogated the interaction of RNF8 and UBCH8, which in turn resulted in compromised ability in promoting K48-based poly-ubiquitylation reactions. Consistent with the pivotal roles of K63-based ubiquitin conjugates in DNA damage signal transduction, we found that UBCH8 depletion did not noticeably affect RNF8 functions in promoting FK2 and 53BP1 IRIF formation (Supplementary Figure S4C), suggesting that the RNF8–UBCH8-catalyzed ubiquitin conjugates composed of K48 chains are not required for the assembly of DNA repair factors at the damaged chromatin.

## DISCUSSION

In this study, we have described a novel intrinsic property of a RING domain that enables differential synthesis

of ubiquitin chains composed of distinct linkages. We found that a subtle point mutation on the DNA damage-responsive E3 ubiquitin ligase RNF8 uncoupled its K48- from its K63-linked ubiquitin chain promoting activities. This was accomplished, in part, via regulation of RNF8 RING-dependent interactions with E2s UBCH8 and UBC13 (Figure 7).

Mutating the conserved isoleucine residue that reside within the zinc-binding loop of RING domain-containing E3 ubiquitin ligases, including MDM2 (I440A), BRCA1 (I26A) and RING1b (I53A), altered their hydrophobic binding surface, and disrupted interactions with their respective E2s (16–19). Built upon our previous work that implicated important roles of electrostatic contribution in selective E3–E2 pairing (25), we found that the RNF8 bearing an I405A mutation on its RING domain selectively perturbed its interaction with UBCH8 and abrogated K48-linked ubiquitylation activities. Interestingly, the RNF8 I405A mutation did not completely abolish its ubiquitin ligase activity. Rather, RNF8 I405A interacted with the E2 ubiquitin conjugating enzyme UBC13 and promoted K63-linked ubiquitin chains. The functionality of the RNF8 I405A–UBC13 interaction is underscored by its ability in catalyzing DNA break-associated ubiquitylation events, which in turn allowed proper assembly of tumor suppressors 53BP1 and BRCA1 at IRIF and promoted cellular survival in response to IR. The fact that the I405A mutation, which is intrinsic to RNF8, specifically disrupted its interaction with UBCH8 but not UBC13 raises the possibility that



**Figure 7.** Differential functions of RNF8 E3 ubiquitin ligase activity. RNF8, via UBC13, catalyzes K63-linked poly-ubiquitylation at DSBs. Ubiquitylated H2A and H2AX are pivotal in assembling downstream DNA damage response proteins including 53BP1 and BRCA1. RNF8 also interacts with UBCH8 to synthesize K48-linked ubiquitin chains. This may occur on RNF8 itself, allowing the E3 ubiquitin ligase to regulate its own abundance in cells, and/or may involve other unknown substrates.

pairing of RNF8–E2s may be subjected to stringent regulation *in vivo*. In line with this possibility, a previous report identified HERC2 as a scaffold protein that promoted DNA damage ubiquitin signaling by enhancing the RNF8–UBC13 association (13). Inactivation of HERC2 weakened the RNF8–UBC13 interaction, and on the other hand, enhanced RNF8 binding to UBCH8 (13). Thus, although it remains unknown mechanistically how HERC2 regulates specific pairing of RNF8 and its E2s, it is tempting to speculate that HERC2, via its binding to RNF8, may alter the RNF8 RING to favor binding to UBC13 but not UBCH8. Further experiments will be needed to address this possibility.

A scenario is now emerging in which the damage-modified chromatin is decorated by a plethora of ubiquitin conjugates, each of which may serve specific functions. In concert, these post-translational modifiers coordinate cell proliferation and DNA repair, and promote optimal cell survival in response to genotoxic stress (9,26). While RNF8 functions involving UBC13-dependent K63-linked ubiquitin chains play important roles in assembling checkpoint and repair proteins at sites of DNA breaks, we did

not observe a requirement for UBCH8 in these events (9) (Supplementary Figure S4C), suggesting that the RNF8–UBCH8 module may regulate yet-to-be identified cellular processes. Apart from mediating histone ubiquitylation events at DNA damage sites, previous studies indicated that RNF8 regulates mitotic exit (27,28). Thus, it will be of significant interest to test whether the RNF8-dependent K48-ubiquitin chains may be important in this regard. Our observations that the subtle point mutation on the RNF8 polypeptide selectively compromised K48- but not K63-ubiquitin chain promoting activities provide a strategic means to specifically dissect RNF8–UBCH8 functions *in vivo*.

Interestingly, while we found that RNF8 RING domain mutants (i.e. I405A and  $\Delta$ RING) displayed enhanced stability when compared to wild-type proteins, turnover of these RING mutants nevertheless was substantial (Figure 5D). We suspect that RNF8 may also be targeted by other E3 ubiquitin ligases. Further experiments will be needed to address this possibility.

In conclusion, we have provided novel insight of how RNF8 may differentially mediate K48- and

K63-poly-ubiquitylation, and have illustrated the versatility of specific E3–E2 pairings in regulating different types of poly-ubiquitin chains. Given that any given E3 ubiquitin ligase may interact with multiple E2s, with each devoted to specific cellular functions, our identification of RNF8 I405A with selective inactivation of K48- but not K63-ubiquitin chain promoting activity also highlights potential methodologies via which to study defined functions involving distinct ubiquitin chain topologies.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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