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RESEARCH ARTICLE

Identification of RNF168 as a PML nuclear body regulator

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ABSTRACT

Promyelocytic leukemia (PML) protein forms the basis of PML nuclear bodies (PML NBs), which control many important processes. We have screened an shRNA library targeting ubiquitin pathway proteins for effects on PML NBs, and identified RNF8 and RNF168 DNA-damage response proteins as negative regulators of PML NBs. Additional studies confirmed that depletion of either RNF8 or RNF168 increased the levels of PML NBs and proteins, whereas overexpression induced loss of PML NBs. RNF168 partially localized to PML NBs through its UMI/MIU1 ubiquitin-interacting region and associated with NBs formed by any PML isoform. The association of RNF168 with PML NBs resulted in increased ubiquitylation and SUMO2 modification of PML. In addition, RNF168 was found to associate with proteins modified by SUMO2 and/or SUMO3 in a manner dependent on its ubiquitin-binding sequences, suggesting that hybrid SUMO–ubiquitin chains can be bound. *In vitro* assays confirmed that RNF168, preferentially, binds hybrid SUMO2–K63 ubiquitin chains compared with K63–ubiquitin chains or individual SUMO2. Our study identified previously unrecognized roles for RNF8 and RNF168 in the regulation of PML, and a so far unknown preference of RNF168 for hybrid SUMO–ubiquitin chains.

KEY WORDS: PML nuclear bodies, RNF168, RNF8, SUMO

INTRODUCTION

Promyelocytic leukemia (PML) nuclear bodies (NBs) are discreet foci in mammalian cells that are based on PML protein (which has several isoforms). The PML protein was so named because a translocation that yields a fusion protein of PML and the retinoic acid receptor α (RARα) protein (PML–RARα) results in the development of acute promyelocytic leukemia (APL) (Salomoni et al., 2008). Considerable evidence now indicates that PML and NBs are tumor suppressors in general, and that increased PML levels can effectively inhibit the growth of tumor cells (Scaglioni et al., 2006; Wu et al., 2014; Salomoni et al., 2008). The tumor-suppressing properties of PML NBs stem from their important contributions to multiple cellular processes, including apoptosis, p53 activation, DNA repair and senescence (Salomoni et al., 2008; Lallemand-Breitenbach and de The, 2010; Nisole et al., 2013).

PML NBs contain six different nuclear PML isoforms (PML I to VI) that are derived from alternative splicing events in the C-terminal part of the protein, and there is evidence that specific PML isoforms mediate distinct functions associated with the NBs (Bernardi and Pandolfi, 2007; Condemine et al., 2006; Nisole et al., 2013). PML contains a SUMO-interacting motif (SIM) and multiple SUMO-modified sites, which mediate interactions between several PML proteins and allow for the formation of a NB (Bernardi and Pandolfi, 2007; Shen et al., 2006b). In addition to PML, NBs contain many other proteins that vary in their dynamics of association and mediate the various NB functions. Many proteins (e.g. Daxx) associate with PML NBs through SUMO–SIM interactions with PML core sequences, whereas others (e.g. p53) associate with C-terminal tail sequences that are specific to a particular isoform (Bernardi and Pandolfi, 2007; Nisole et al., 2013).

In addition to their cellular roles, PML NBs are part of the innate immune response that suppresses infection by several viruses (Geoffroy and Chelbi-Alix, 2011; Everett and Chelbi-Alix, 2007; Nisole et al., 2013; Tavalai and Stamminger, 2011; Sivachandran et al., 2012b). To overcome PML suppression, these viruses encode proteins that disrupt PML NBs through a variety of mechanisms that include PML degradation, interference with PML interaction by inhibiting SUMOylation and restructuring of PML NBs into tracts (Tavalai and Stamminger, 2009; Nisole et al., 2013; Geoffroy and Chelbi-Alix, 2011; Everett and Chelbi-Alix, 2007; Sivachandran et al., 2008). These studies have shown that PML NB function can be regulated by manipulation of PML and have provided a framework for studies on cellular regulation of PML NBs. In addition, considerable information on the regulation of PML NBs has come from studying how they are affected by As_2O_3, a treatment for APL, which induces the loss of NBs formed by PML–RARα fusion proteins that allows restoration of NBs formed by the non-mutated copy of PML (Ablain et al., 2014; de The et al., 2012). Arsenic (As) induces hyper-SUMOylation of PML, resulting in recruitment of the SUMO-targeted E3 ligases RNF4 and Arkadia (also known as RNF111) (Erker et al., 2013; Lallemand-Breitenbach et al., 2008; Tatham et al., 2008). RNF4 in particular has been shown to be important for the As-induced PML degradation (Lallemand-Breitenbach et al., 2008; Tatham et al., 2008).

Under normal cell growth conditions, the protein level of PML also appears to be largely regulated by ubiquitylation and proteasomal-mediated degradation (Rabellino and Scaglioni, 2013). PML contains a phosphodegron that, upon phosphorylation by casein kinase 2 (CK2), triggers polyubiquitylation (Scaglioni et al., 2006, 2008). However, the E3 ligase responsible for this ubiquitylation has not been identified. A few ubiquitin E3 ligases have been reported to promote loss of PML under specific circumstances. For example, the KLHL20–Cul3–ROC1 complex downregulates PML in response to hypoxia (Yuan et al., 2011), and SIAH1 and SIAH2 can induce PML degradation upon overexpression (Fanelli et al., 2004). In addition, E6AP has been identified as a PML regulator in Burkitt's lymphoma (Wolyniec et al., 2012). The ubiquitin-specific protease USP7 has also been shown to negatively regulate PML NBs in nasopharyngeal carcinoma cells by promoting the degradation of PML in a manner

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that is independent of its catalytic activity (Sarkari et al., 2011; Sivachandran et al., 2008). Despite these specific studies, there is still much to be learned about which ubiquitin pathway proteins regulate the level of PML proteins and NBs.

To gain a more comprehensive understanding of cellular ubiquitin pathway proteins that regulate PML NBs, we screened an shRNA library targeting ~500 ubiquitin pathway proteins for effects on PML NBs. The screen identified RNF8 and RNF168 which are known to work together in DNA repair, as E3 ubiquitin ligases whose depletion increases the number and intensity of PML NBs. Additional studies confirmed that silencing of RNF8 or RNF168 increased PML NBs and protein levels, whereas overexpression of either protein induced loss of PML NBs. We further showed that RNF168 localized to PML NBs through the UIM- and MIU-related ubiquitin-binding domain (UMI) and motif interacting with ubiquitin 1 (MIU1), hereafter referred to as UMI/MIU1, sequence in a PML isoform-independent manner and binds to hybrid SUMO2–ubiquitin chains. The results suggest additional roles for RNF8 and RNF168 in PML regulation.

RESULTS

Identification of RNF8 and RNF168 as PML regulators

We have previously identified a mechanism by which the Epstein-Barr virus (EBV) protein EBNA1 induces loss of PML NBs and degradation of PML proteins in nasopharyngeal carcinoma (NPC) and gastric carcinoma, two EBV-associated cancers (Sivachandran et al., 2012a, 2008, 2010). However, little is known about the cellular ubiquitin pathway proteins that regulate PML in these cells. To identify cellular ubiquitin pathway proteins that regulate PML NBs in NPC cells, we conducted a high-content screen in which ~2500 lentiviruses express shRNAs that had been designed to silence ~500 ubiquitin pathway proteins – ubiquitin-activating, ubiquitin-conjugating and ubiquitin-ligating (E1, E2 and E3, respectively) enzymes, as well as deubiquitylating proteins – were used to infect the NPC cell line CNE2Z in triplicate. Several days later, cells were stained for PML, imaged by using confocal microscopy, and the number and intensity of PML NBs was determined. Target proteins in which two or more of the five shRNAs increased the average PML NB number 1.5-fold or more, or increased the average PML NB intensity 2-fold or more were considered for further study. The E3 ligases RNF8 and RNF168, which are known to work together to repair double-stranded DNA breaks (Al-Hakim et al., 2010), met these criteria. As shown in Fig. 1, multiple shRNAs targeting RNF8 or RNF168 increased the number and/or intensity of PML NBs relative to non-infected cells and also to a negative control lentivirus-expressed shRNA targeting RNF111. The latter lentivirus served as a negative control for lentivirus infection because RNF111 expression levels were already undetectable in these cells (data not shown).

The effect of RNF8 and RNF168 on PML NBs was further examined by depleting these proteins with small interfering RNA (siRNA). siRNA-mediated depletion of either protein resulted in an obvious increase in the number and intensity of the PML NBs (Fig. 2A and B). In keeping with these results, western blots showed that the total level of PML was increased upon silencing RNF8 or RNF168 (Fig. 2C). siRNA-mediated depletion of RNF8 or RNF168 also increased the levels of PML NBs and proteins in U2OS cells, showing that the effects are not cell line specific (Fig. 2D to F). Conversely, overexpression of FLAG-tagged RNF8 or RNF168 decreased the number of PML NBs (Fig. 3).

RNF168 can modulate PML NBs independently from RNF8

In response to DNA damage, RNF8 is recruited to sites of double-stranded DNA breaks (DSBs) where it ubiquitylates histones H2A and/or H2AX, which results in recruitment of RNF168. Therefore, the role of RNF168 in the DNA damage response (DDR) depends on RNF8. We asked whether a similar relationship exists for the roles of RNF8 and RNF168 in regulating PML NBs. To this end, we treated CNE2Z cells with siRNA targeting RNF8, or with negative control siRNA, then transfected them with a plasmid expressing FLAG-RNF168 (to overexpress RNF168) or an empty plasmid (pcDNA3). Cells were then stained for FLAG and PML, and the number of PML NBs in FLAG-positive cells was compared to that of empty-plasmid-treated control cells (Fig. 4). As expected, RNF8

A

Lentivirus	PML bodies/cell	Intensity/NB
none	10.4 +/- 2.2	124.2 +/- 20
RNF111	11+/-3.0	122+/-19
RNF168-1	14.5 +/- 1.7	282 +/-38
RNF168-2	11.8 +/-1.5	180 +/-10
RNF168-3	11.8 +/-1.5	149+/-27
RNF168-4	19.5 +/-1.3	417+/-39
RNF168-5	13.5 +/- 1.3	167+/-20
RNF8-1	18.5+/-2.1	218 +/-9.9
RNF8-2	8.0 +/-2.2	235 +/-30
RNF8-3	10.3 +/-3.1	154 +/-18
RNF8-4	18.0 +/-4.8	172 +/-16
RNF8-5	13.0 +/-3.8	196 +/-55

B

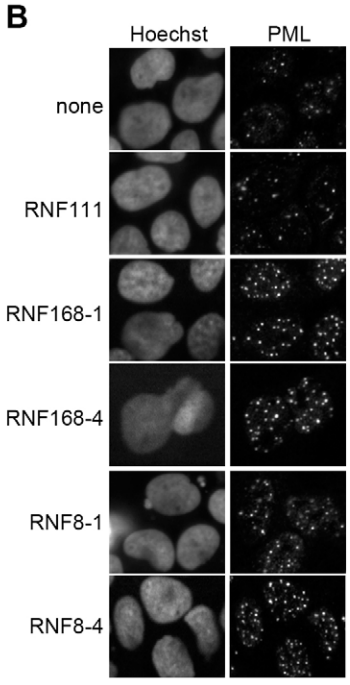

Fig. 1. An shRNA screen identifies RNF8 and RNF168 as regulators of PML NBs. CNE2Z cells were infected with lentivirus expressing shRNA targeted against RNF111 (negative control), RNF168 (shRNAs 1 to 5) or RNF8 (shRNAs 1 to 5) or left uninfected (none). Cells were then fixed, stained with Hoechst dye 33342 and with PML-specific antibody, then imaged by confocal microscopy. (A) The number of PML NBs per cell and intensity of PML NBs was determined. Average values with standard deviation are shown. (B) Sample images of cells infected with lentivirus expressing the indicated shRNAs listed in A.

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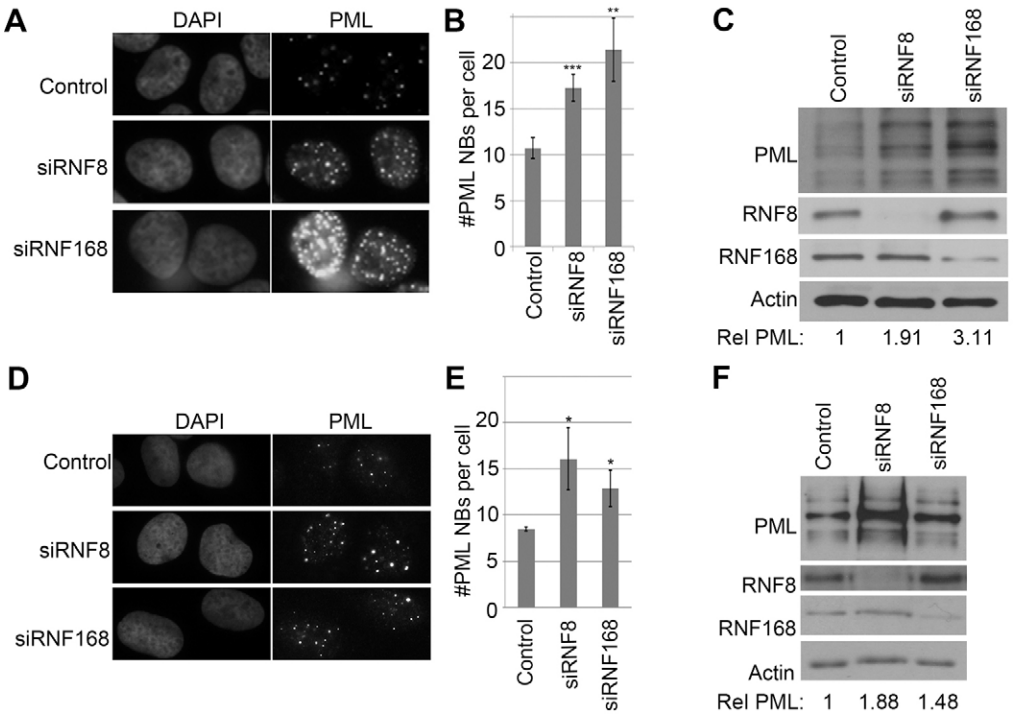

Fig. 2. siRNA targeting RNF8 and RNF168 increases PML NBs and PML protein levels. (A) CNE2Z cells were transfected with siRNA targeting RNF8 or RNF168, or with negative control siRNA (control), then stained for PML. (B) The number of PML NBs per cell was determined for 100 cells in three independent experiments and average values with standard deviation were plotted. (C) CNE2Z cells treated with siRNA as in A were lysed and analyzed by western blotting using the indicated antibodies. PML bands were quantified by densitometry and normalized to actin, and values for each lane are shown relative to the Control lane under the gel. (D–F) The same experiment as in A–C but performed in U2OS cells. *P* values (determined by *t*-tests) are indicated as follows: **P*<0.05; ***P*<0.01; ****P*<0.001.

silencing (which was confirmed by western blotting; Fig. 4B) in the presence of pcDNA3 resulted in a notable increase in the number of PML NBs (from 11 to 16 on average; Fig. 4A and C). Also, as expected, in control siRNA samples RNF168 overexpression notably decreased the number of PML NBs relative to the pcDNA3 control (from 11 to 6 on average; Fig. 4A and C). However, RNF168 overexpression was also found to decrease PML NBs in RNF8-silenced samples (from 16 to 9 on average; Fig. 4A and C). The results indicate that RNF168 is able to decrease PML NBs in the absence of RNF8, at least under conditions of RNF168 overexpression. For completeness, we also did the opposite experiment, in which the ability of overexpressed RNF8 to induce PML NB loss was tested with and without silencing of RNF168 (Fig. S1). RNF8 retained the ability to induce a decrease of PML NBs after RNF168 depletion, indicating that RFN8 and RNF168 can work independently of each other. We focused our further studies on RNF168.

Localization of RNF168 to PML NBs

To gain insight into whether RNF168 acts directly at PML NBs, we asked whether RNF168 localized to PML NBs. To this end, FLAG-tagged RNF168 constructs with and without deletions of

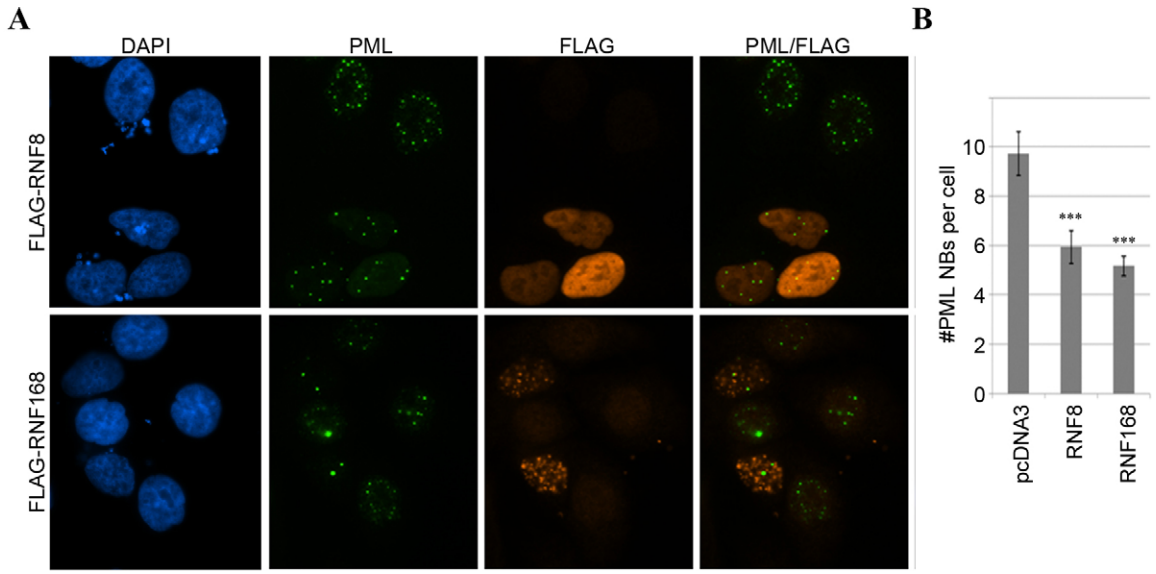

Fig. 3. Overexpression of RNF8 or RNF168 induces loss of PML NBs. (A) CNE2Z cells were transfected with plasmids expressing FLAG-tagged RNF8 or RNF168 and then stained for FLAG and PML. Sample images showing fewer PML NBs in FLAG-positive cells. (B) PML NBs were counted in cells expressing FLAG-RNF8 or FLAG-RNF168 and compared to cells transfected with empty plasmid (pcDNA3). Average values with standard deviation are shown from three independent experiments. *P* values (determined by *t*-tests) are indicated as follows: ****P*<0.001.

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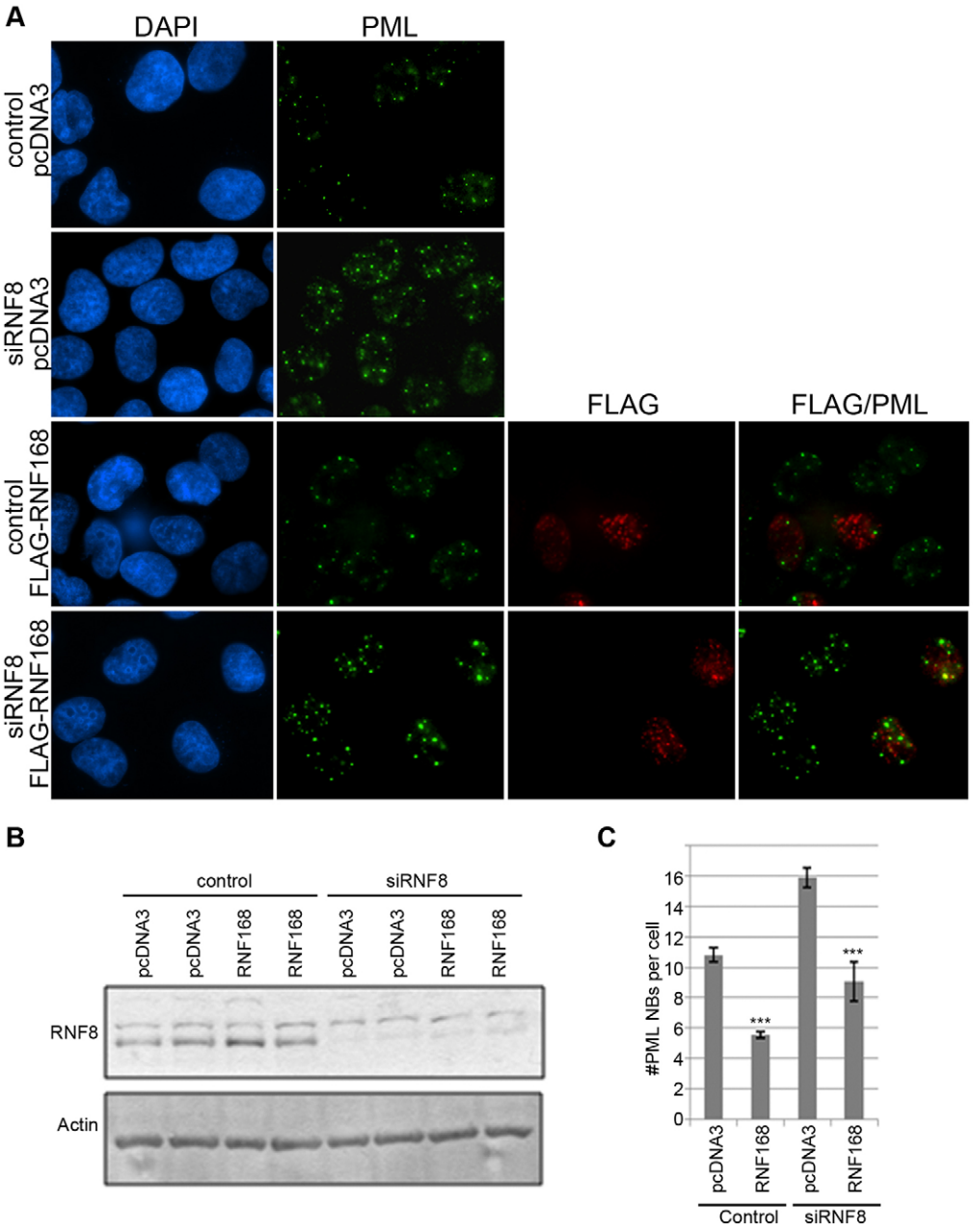

Fig. 4. RNF168 can induce loss of PML NBs independent of RNF8. CNE2Z cells were treated with siRNA targeting RNF8 (siRNF8) or with negative control siRNA (control) and then transfected with the FLAG-RNF168 expression plasmid (bottom two rows in A) or empty control plasmid pcDNA3 (top two rows in A). Cells were then either fixed and stained for PML and FLAG (A) or lysed and analyzed by western blotting using RNF8 specific antibody to confirm RNF8 silencing (B). (C) PML NBs in A were counted in 100 cells of three independent experiments. For RNF168 samples, PML NBs were counted only in FLAG-positive cells. Average values with standard deviations were plotted with *P* values (***$P<0.001$) for RNF168 overexpression shown relative to the pcDNA3 control for each condition.

previously characterized functional elements were expressed in CNE2Z cells, followed by staining for FLAG and PML NBs (Fig. 5A and B). As previously reported, overexpression of WT RNF168 resulted in the formation of aggregates that interfered with its assessment of nuclear localization (Pinato et al., 2009). This aggregation was not seen when using RNF168 mutants that lack either the RING domain (ΔRING) or MIU2 ubiquitin-binding sequence (ΔMIU2). Both of these proteins showed diffuse nuclear staining with some foci that localized to the PML NBs (Fig. 5B). The localization to PML NBs was not an artifact of overexpression because ΔRING localized to PML NBs even at very early post-transfection times (6 h post-transfection), when ΔRING expression levels were close to those of endogenous RNF168 (Fig. S2A). The localization of RNF168 PML NBs was not particular to CNE2Z cells because ΔMIU2 was also found to form foci in U2OS cells, some of which coincided with PML NBs (Fig. S2B). Localization of RNF168 to PML NBs was abrogated when the UMI and MIU1 ubiquitin-binding sequences were deleted individually or together within the context of ΔMIU2 (compare ΔMIU2 to Δ100-201ΔMIU2, ΔMIU1ΔMIU2 or ΔUMIΔMIU2 in Fig. 5B), implying that the UMI/MIU1 region is a PML-targeting sequence. Although the expression levels of the different mutants examined for PML localization varied considerably (Fig. 5C), there was no correlation between PML NB localization and expression level. The ability of the UMI/MIU1 region to interact with PML NBs was further examined by expressing an RNF168 fragment containing amino acids 100–201 fused to GFP (GFP-100-201) (Fig. 5C and D). GFP-100-201 formed prominent foci that localized to PML NBs, whereas no such foci were seen with the expression of GFP alone (Fig. 5D). In addition, removal of the MIU1 region from this construct (GFP-100-166) resulted in the loss of prominent foci that localized to PML NBs, confirming that MIU1 plays a role in the recruitment to PML NBs.

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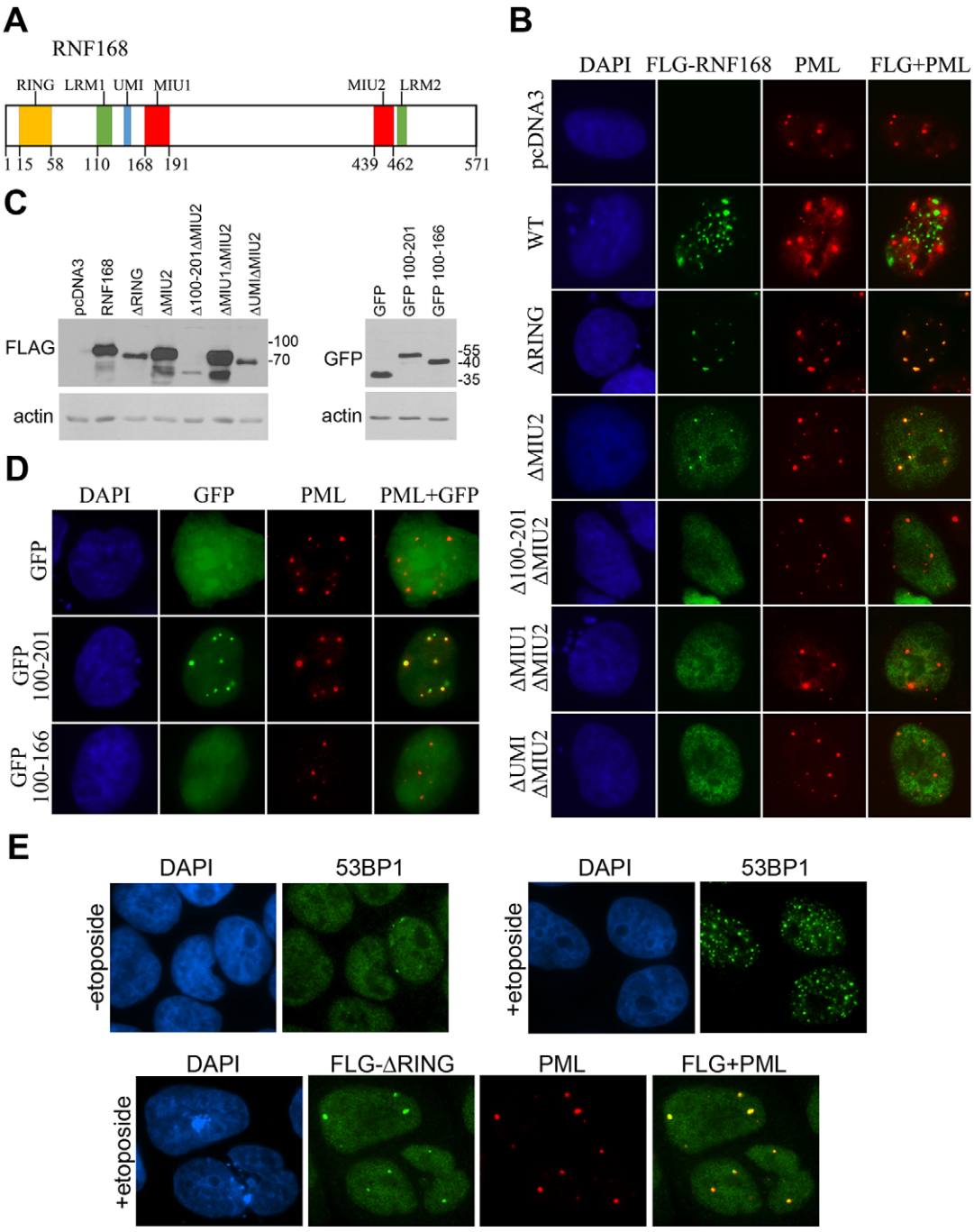

Fig. 5. RNF168 localization to PML NBs. (A) Schematic representation of RNF168 showing the positions of the catalytic RING domain, ubiquitin-binding sequences (UMI, MIU1, MIU2) and LR motifs (LRM) (Panier et al., 2012). (B) CNE2Z cells were transiently transfected with plasmids expressing FLAG-tagged wild-type RNF168 (WT), with FLAG-tagged RNF168 of the indicated deletions or with pcDNA3 empty plasmid. Cells were then stained for FLAG and PML, and imaged by fluorescence microscopy. (C) Western blots for FLAG-tagged and GFP-tagged proteins as described for B and D. (D) CNE2Z cells were transfected with plasmids expressing GFP fused to RNF168 amino acids 100–201 (containing LRM1, UMI and MIU1), amino acids 100–166 (containing LRM1 and UMI) or GFP alone, and then stained for PML and imaged for GFP and PML. (E) Bottom panels: CNE2Z cells were transiently transfected with plasmids expressing FLAG-ΔRING, treated with etoposide (10 µg/ml) for 5 h, then stained for FLAG and PML, and imaged using fluorescence microscopy. Top panels: CNE2Z cells were treated with etoposide as described for bottom panels or left untreated as indicated, then stained for 53BP1 to confirm that DDR foci were induced by etoposide.

Because RNF168 is known to have a role in DDR involving its recruitment to DNA repair sites, we examined whether RNF168 can also associate with PML NBs during DDR. To this end, FLAG-ΔRING was expressed in CNE2Z cells that were then treated with the topoisomerase inhibitor etoposide to induce DDR (as evidenced by the formation of 53BP1; Fig. 5E top panels), followed by staining for FLAG and PML. As shown in Fig. 5E, ΔRING was still detected at PML NBs, indicating that RNF168 retains its ability to associate with PML NBs during DDR. This result is similar to those described in studies that investigated the localization of the Bloom (BLM) DDR protein, which localizes to PML NBs both before and after induction of DNA damage (Bischof et al., 2001).

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PML NBs contain six different PML isoforms that vary in length and sequence of their C-terminal regions. Some proteins associate with PML NBs through isoform-specific sequences, whereas others associate with the conserved PML core sequence. To determine whether a specific PML isoform is responsible for RNF168 recruitment to PML NBs, we examined the localization of RNF168 ΔMIU2 in CNE2Z cells that express single PML isoforms. These cells had been previously generated by silencing total PML with lentivirus-delivered shRNA, then adding back a silencing-resistant PML isoform with a second lentivirus (Sarkari et al., 2011; Cuchet et al., 2011). Each PML isoform forms NBs and this system has been previously used to reveal isoform specificities of some viral and cellular proteins (Sarkari et al., 2011; Sivachandran et al., 2012b; Cuchet-Lourenco et al., 2012). Examination of the localization of RNF168 ΔMIU2 in cells with single PML isoforms, revealed that this protein can localize to NBs formed by any PML isoform, indicating that interaction is not through the C-terminal tail of PML (Fig. 6).

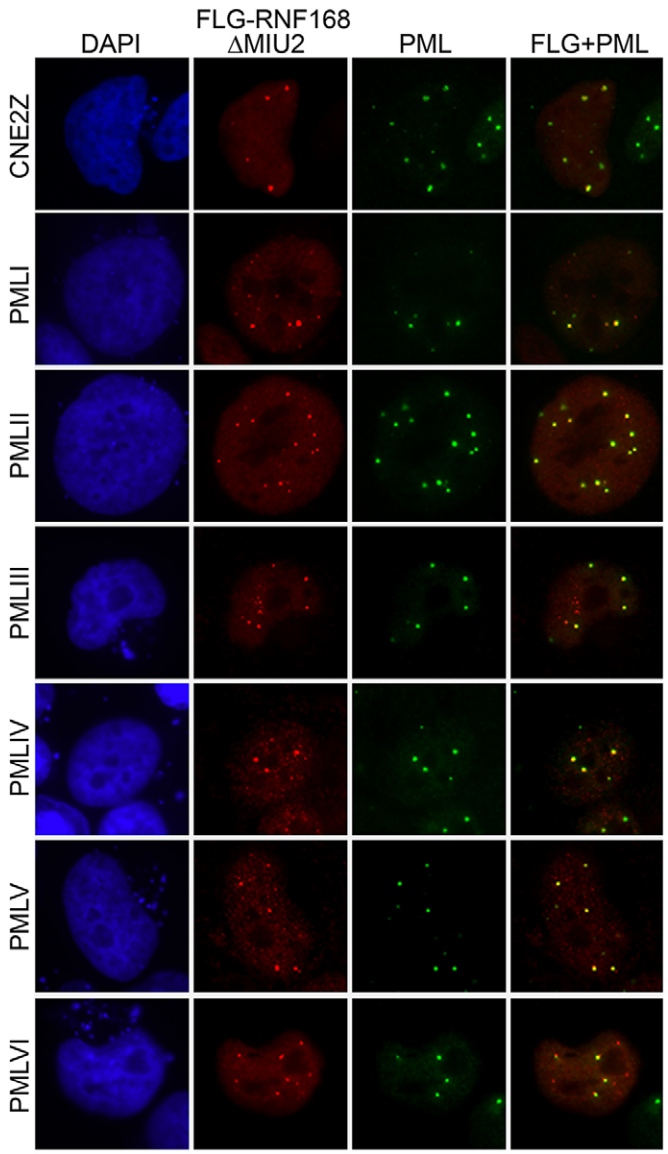

Fig. 6. RNF168 localizes to NBs formed by any PML isoform. FLAG-tagged RNF168 ΔMIU2 was expressed in wild-type CNE2Z cells (CNE2Z) or in CNE2Z cells that lack WT PML but express a single recombinant PML isoform (PMLI, PMLII, PMLIII, PMPIV, PMLV, PMLVI) (Sarkari et al., 2011; Sivachandran et al., 2012b). Cells were then stained for PML, and FLAG (FLG) plus PML. Nuclei were stained using DAPI.

RNF168 increases PML ubiquitylation and SUMOylation

The loss of PML NBs can result from polyubiquitylation and degradation of PML or from interference with SUMOylation of PML, which interferes with the ability of PML to interact to form NBs. Therefore, we examined the effect of RNF168 overexpression on the ubiquitylation and SUMOylation of PML (Fig. 7). To this end, CNE2Z cells were co-transfected with a plasmid expressing HA-tagged ubiquitin and a second plasmid that expressed FLAG-tagged RNF168, an RNF168 mutant or empty plasmid, and then treated with proteasomal inhibitor (MG132) to inhibit degradation of the ubiquitylated proteins. PML was immunoprecipitated (IP) with antibody recognizing all PML isoforms under denaturing conditions, and then immunoblotted for PML and HA (Fig. 7A). Both full-length and ΔMIU2 versions of RNF168 were found to induce the accumulation of polyubiquitylated forms of PML, whereas the results with an RNF168 mutant that lacks all three ubiquitin-interacting motifs (ΔUMIΔMIU1ΔMIU2; hereafter called ΔUb) resembled the results with the empty plasmid negative control. Therefore, the sequences that are necessary for RNF168 to associate with PML NBs are also needed for RNF168 to induce PML ubiquitylation. An RNF168 mutant lacking the catalytic RING domain (ΔRING) was also tested for the induction of PML ubiquitylation and found to be impaired relative to RNF168 or ΔMIU2. However, the very low expression levels of this mutant relative to other RNF168 proteins makes this result inconclusive.

To further examine the nature of PML modifications through ubiquitin that are induced by RNF168, we repeated the above experiment in the presence and absence of MG132; in addition to detecting total ubiquitin (HA blot), we also blotted with antibodies specific for K48- and K63-linked ubiquitin chains (Fig. 7B). As expected, MG132 treatment resulted in increased levels of total ubiquitylated products (HA blot; input lanes) and K48-linked ubiquitin products (K48 blot; input lanes). In addition, a smaller increase in K63-linked ubiquitin products (K63 blot; input lanes) was detected in the presence of MG132, consistent with reports that hybrid chains that contain SUMO2 and K63 ubiquitin can trigger proteasomal degradation (Tatham et al., 2011). Consistent with the known role of RNF168 in synthesizing K63 ubiquitin chains, the total amount of these chains (in input lanes) was increased when RNF168 was overexpressed (relative to the pcDNA3 control lane). PML IP experiments showed induction of PML ubiquitylation (detected in the HA blot) by RNF168 and ΔMIU2 (relative to the control lane) only when proteasomal degradation was blocked, consistent with a role of this ubiquitylation in PML degradation. Immunoprecipitated PML showed some reactivity with both antibodies against K48 and K63 ubiquitin under all conditions, but RNF168 and ΔMIU2 were found to increase levels of K48 ubiquitin (and only to a lesser degree of K63 ubiquitin) on PML (compared to control lanes) when the proteasome was blocked. Together, the results suggest that RNF168 can increase the levels of both K48 and K63 ubiquitin chains on PML.

PML is modified by the addition of SUMO1, SUMO2 and SUMO3. These modifications are needed for NB formation, whereas hyper-SUMOylation can be a step in the degradation process of PML (as induced by As_2O_3). We examined the effect of RNF168 overexpression on PML SUMOylation by co-transfecting CNE2Z cells with a plasmid expressing Myc-tagged SUMO2 and the indicated FLAG-tagged RNF168 protein or empty plasmid, followed by IP of total PML (Fig. 7C). Relative to the empty

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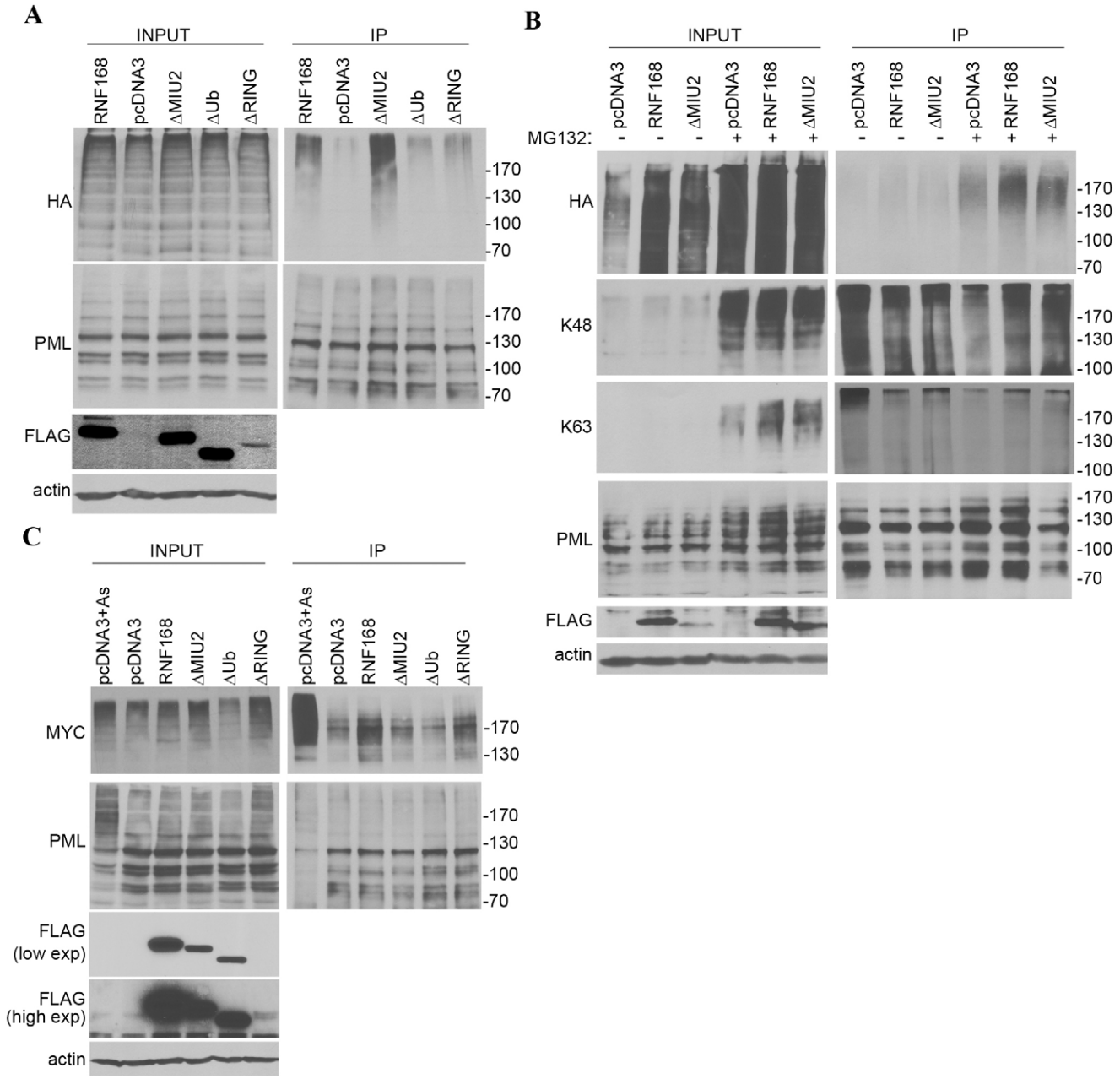

Fig. 7. RNF168 induces PML ubiquitylation and SUMOylation. (A) CNE2Z cells were transfected with a plasmid expressing HA-tagged ubiquitin and a second plasmid expressing RNF168 or the RNF168 mutants ΔMIU2, ΔUb (ΔUMIΔMIU1ΔMIU2) or ΔRING or empty plasmid (pcDNA3) in the presence of proteasome inhibitior MG132. Total PML was immunoprecipitated from cell lysates and analyzed by western blotting for PML and HA (IP). A sample of the cell lysate prior to immunoprecipitation is also shown (INPUT). (B) Experiments were performed as described for A in the presence (+) or absence (–) of MG132. Ubiquitylated proteins in INPUT- and IP-labeled lanes were detected by western blotting using antibodies against HA, K48-linked ubiquitin or K63-linked ubiquitin. (C) Same experiment as described for A, except that Myc-tagged SUMO2 was expressed instead of HA-tagged ubiquitin. In addition, a positive control for PML SUMOylation is shown, in which cells transfected with pcDNA3 were treated with As_2O_3 (pcDNA3+As). Two exposures of the INPUT FLAG blot are shown. The positions of molecular mass markers are indicated on the right (in kDa).

plasmid negative control, RNF168 was consistently found to increase the levels of SUMO2-modified PML, whereas ΔUb did not exhibit this effect, and ΔMIU2 and ΔRING had intermediate effects. The results suggest that RNF168-induced loss of PML NBs is not due to interference with PML SUMOylation but, rather, that RNF168 can increase PML SUMOylation. In addition, the fact that the low-expressing ΔRING still induced PML SUMOylation to some degree, suggests that the increase in PML SUMOylation is independent of the ubiquitylation activity of RNF168.

RNF168 associates with hybrid SUMO2–ubiquitin chains

The findings that RNF168 associates with PML core sequences – which are known to be SUMOylated and might increase SUMOylation of PML – prompted us to further examine the ability of RNF168 to bind SUMO. To this end, CNE2Z cells were co-transfected with a plasmid expressing Myc-tagged SUMO1, SUMO2 or SUMO3 and a plasmid expressing FLAG-tagged RNF168, ΔUb or empty plasmid. FLAG-RNF168 was recovered by IP and recovery of Myc-SUMO examined by western blots

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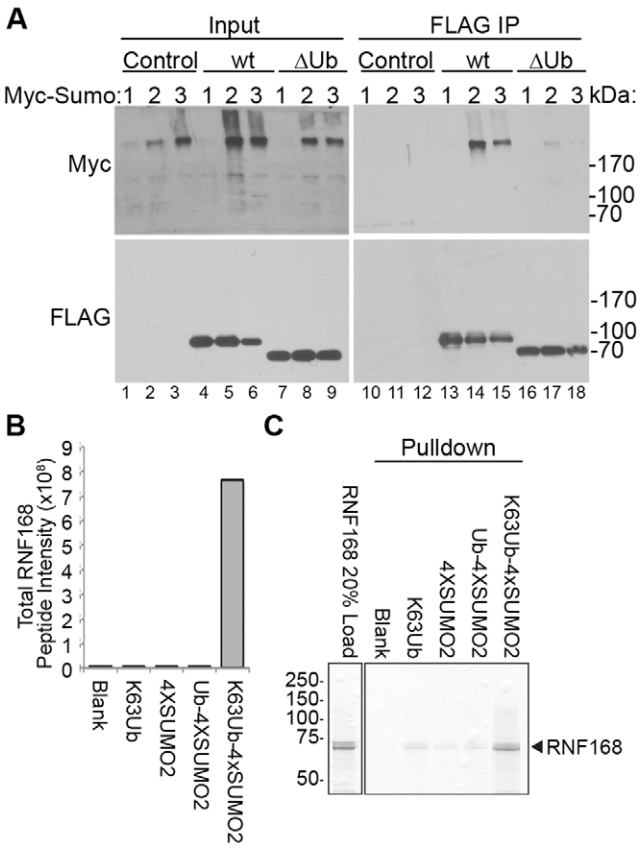

Fig. 8. RNF168 associates with SUMO2- and/or SUMO3-modified proteins in cells and preferentially binds ubiquitin–SUMO hybrid chains *in vitro*. (A) 293T cells were transfected with plasmids expressing FLAG-tagged RNF168 (wt), RNF168ΔUb (ΔUMIΔMIU1ΔMIU2) or with empty plasmid (Control), together with a second plasmid expressing Myc-tagged SUMO1, SUMO2 or SUMO3. After cell lysis, a sample was analyzed by western blotting (Input), while the remaining sample was subjected to FLAG IP, followed by western blotting for Myc and FLAG. The positions of molecular mass markers are indicated on the right (in kDa). (B) Comparison of retention of RNF168 from 293 cell extracts on affinity resins containing K63-ubiquitin polymers (K63-Ub), a linear 4xSUMO2 chain (4xSUMO-2), an N-terminally monoubiquitinated 4xSUMO2 chain (Ub-4xSUMO-2), hybrid chains containing K63-ubiquitin polymers linked to 4xSUMO-2 (K63Ub-4xSUMO-2) or no protein (blank). Total intensity of RNF168 peptides retained on each resin is shown. RNF168 identification was based on 26 unique peptides; sequence coverage was 47%. (C) Purified RNF168 was incubated with the affinity resins or blank resin from the experiments described in B and, after washing was eluted with denaturing buffer. A Coomassie-Blue-stained gel is shown of the protein loaded onto the resins (load) and eluted from the indicated resins (Pulldown).

(Fig. 8A). Consistent with previous reports, input lanes showed that SUMO2 and SUMO3 formed chains of high molecular mass more efficiently than SUMO1. In addition, an increase in the total levels of SUMO2 or SUMO3 chains was consistently seen in the presence of RNF168 when compared to the empty plasmid control (compare lane 2 to lane 5, and lane 3 to lane 6). FLAG IPs showed that full-length RNF168 associates with the SUMO2 and SUMO3 chains (compare lanes 11 and 12 to lanes 14 and 15), and that this interaction is greatly decreased by deletion of the three ubiquitin-interacting regions in the ΔUb mutant (lanes 17 and 18). We did not detect any shifted forms of FLAG-RNF168 in either the input or FLAG-IP samples (see FLAG blots), suggesting that RNF168 itself is not SUMO-modified but, rather, can bind to SUMO2 and/or SUMO3 chains. However, a direct interaction between purified RNF168 and free SUMO2 or SUMO3 chains was not detected in *in vitro* binding assays (data not shown). This suggests that RNF168 is not simply recognizing SUMO2 and/or SUMO3 but, rather, that is recognizes SUMO2 and/or SUMO3 in a particular context.

Hybrid SUMO–ubiquitin chains that consist of a SUMO2 chain joined to a K63-linked ubiquitin chain have been described (Tatham et al., 2013), and can be specifically recognized by some proteins (Geoffroy and Hay, 2009; Guzzo et al., 2012; Hu et al., 2012). The UMI and/or MUI1 sequences of RNF168 that we found to be important for recovery of SUMO2 chains have been previously shown to recognize K63-linked ubiquitin (Panier et al., 2012), raising the possibility that the SUMO2-containing material that we recovered are hybrid SUMO–ubiquitin chains. To investigate this possibility, affinity resins were generated that contain one of the following: K63-ubiquitin polymers (K63-Ub), linear 4xSUMO2 chains (4xSUMO-2), N-terminally monoubiquitinated 4xSUMO2 chains (Ub-4xSUMO2) or hybrid chains containing K63-ubiquitin polymers linked to 4xSUMO2 (K63Ub-4xSUMO2) (see Fig. S3A). Nuclear extracts from 293 cells were incubated with each of these resins as well as with empty resin (blank), and proteins retained on each resin were identified and quantified by mass spectrometry-based label-free quantitative proteomics (Fig. S3B). Recovery ratios of each protein on a particular SUMO and/or ubiquitin resin relative to the blank resin were then determined (Fig. S3C and D). RNF168 was one of the highest scoring proteins, with much higher recovery on resin containing K63Ub-SUMO2 hybrid chains than on any of the other resins (Fig. 8B, Fig. S3C and D).

To determine whether RNF168 binds directly to the hybrid chains, assays of the above affinity resins were repeated using purified RNF168 generated in *E.coli*; eluted proteins were analyzed by SDS-PAGE and Coomassie-Blue staining. As shown in Fig. 8C, RNF168 was recovered most efficiently on the resin containing the hybrid K63Ub-4xSUMO2 chains. Consistent with previous reports (Panier et al., 2012), some binding to resin that contained K63-linked polyubiquitin was also detected. The results indicate that the association of RNF168 with SUMO2 chains involves direct binding to hybrid SUMO2–ubiquitin chains.

DISCUSSION

Our screen for proteins that regulate the level of PML NBs identified RNF8 and RNF168 as proteins whose depletion results in increased number and intensity of PML NBs and increased levels of PML proteins. By contrast, overexpression of RNF8 or RNF168 can lead to loss of PML NBs. To date, RNF8 and RNF168 are known for their important roles in the DDR to double-stranded DNA breaks (Bartocci and Denchi, 2013; Al-Hakim et al., 2010). K63-linked ubiquitylation of histones H2A and/or H2AX and/or other proteins by RNF8 at DSBs results in the recruitment of RNF168, which interacts with the ubiquitylated histones through its three ubiquitin-binding motifs (UMI, MIU1, MIU2) (Stewart et al., 2009; Bartocci and Denchi, 2013; Panier and Durocher, 2009; Panier et al., 2012; Mailand et al., 2007; Doil et al., 2009). Further ubiquitylation of H2A and/or H2AX by RNF168 and RNF8 then leads to recruitment of additional DNA-repair proteins, including 53BP1 and BRCA1. Our current studies indicate additional roles for RNF8 and RNF168 as negative regulators of PML NBs.

Here, we have shown that RNF8 and RNF168 can downregulate PML NBs and proteins. We focused additional studies on RNF168 due to its partial colocalization with PML NBs, which suggests it is acting directly on the PML NBs. Overexpression of full-length RNF168 is known to aggregate in cells and, hence, is not typically used for functional studies (Pinato et al., 2009). However, deletion of MIU2 or the RING domain abrogates this

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aggregation and reveals prominent foci that correspond to PML NBs. Analyses of additional deletion mutants and GFP-fusion proteins identified the UMI/MIU1 region as important for PML targeting. Since all of our UMI/MIU1 deletion constructs lack MIU2, we do not know whether PML targeting is unique to UMI/MIU1 or can also be mediated by MIU2.

The mechanism by which RNF168 induces loss of PML NB appears to involve ubiquitylation of PML because overexpression of RNF168 or ΔMIU2 resulted in increased levels of ubiquitylated PML. The effect on PML ubiquitylation also fits with the increase in PML protein levels that were observed upon RNF168 depletion. Induction of PML ubiquitylation requires UMI/MIU1 PML-targeting sequences in RNF168 (that had been deleted in the ΔUb mutant used in experiments shown in Fig. 7), consistent with a requirement to associate with the PML NB in order to induce PML degradation. It remains unclear whether PML is ubiquitylated directly by RNF168, although this would be the simplest interpretation. Interestingly, Tikoo et al. (2013) have previously found that RNF8 can conjugate K63-linked ubiquitin to PML *in vitro*, although this leads to an increase rather than a loss of PML NBs in their system. We also observed that RNF168 but not the ΔUb mutant increases the level of SUMO2-modified PML. SUMO modifications of PML are also factors in the stability of PML. For example, As_2O_3 triggers the loss of PML by first inducing their hyperSUMOylation (Lallemand-Breitenbach et al., 2008, 2001; Tatham et al., 2008). Therefore, the effect of RNF168 on PML SUMOylation might also be a factor in its ability to regulate the level of PML NBs.

RNF168ΔMIU2 was found to localize to NBs formed by any single PML isoform. The PML isoforms are identical except for their C-terminal tails, which differ in length and sequence, and have been shown to mediate some isoform-specific interactions (Cuchet-Lourenco et al., 2012; Sivachandran et al., 2012b; Nisole et al., 2013). The fact that RNF168 localization to PML NBs does not depend on the PML isoform indicates that recruitment of RNF168 to PML NBs involves the PML core sequences. These core sequences contain SUMO-interacting motifs (SIMs) and can be modified by SUMO and, proteins that interact with PML core sequences, typically do so through SUMO–SIM interactions. This prompted us to examine whether RNF168 is SUMOylated or can bind proteins that have been modified by SUMO (Fig. 8). Although RNF168 can be modified by SUMO1 in response to DNA damage (Danielsen et al., 2012), we did not detect any SUMO modifications of RNF168 under our conditions. Rather, RNF168 was found to bind complexes of high molecular mass containing SUMO2 and SUMO3 chains. These interactions were dependent on the presence of the UMI/MIU1 and/or MIU2 ubiquitin-interacting sequences, suggesting either that these sequences can also interact with SUMO2 and/or SUMO3 chains or that the SUMO2 and/or SUMO3 chains that were bound are hybrid chains that also contain ubiquitin.

To clarify whether or not RNF168 can bind hybrid SUMO–ubiquitin chains, we conducted *in vitro* assays comparing recovery of RNF168 on resin that contained chains of SUMO2, K63 ubiquitin or K63Ub-SUMO2 hybrids. We found that the hybrid chains were most efficiently bound by RNF168, whereas binding of purified RNF168 to SUMO2 chains was not detected (Fig. 8B,C and additional unpublished data obtained by us). This suggests that the SUMO2- and/or SUMO3-modified proteins we had recovered from cells contain hybrid SUMO–ubiquitin chains. This would explain the requirement for the ubiquitin-binding sequences of RNF168 and suggests that RNF168 also contains a weak SIM.

Taken together, our data suggest a model in which RNF168 is recruited to PML NBs through direct interaction with hybrid SUMO–ubiquitin chains that are on PML itself or other PML-NB constituents, such as Daxx, Sp100 and BLM, which are also SUMO-modified (Seeler and Dejean, 2001). In this respect, RNF168 might act downstream of RNF4, a SUMO-targeted ubiquitin ligase that generates hybrid chains and is known to negatively regulate PML NBs (Geoffroy and Hay, 2009). Once recruited to PML NBs, RNF168 causes increased ubiquitylation and SUMOylation of PML, which stimulates their proteasomal degradation. It remains to be determined whether RNF168 is recruited to PML NBs through interactions with hybrid SUMO–ubiquitin chains on PML. Conducting experiments in cells that express PML mutants lacking SUMO-modified sites might be one way of clarifying whether SUMO or SUMO–ubiquitin chains on PML are required for RNF168 recruitment and induced ubiquitylation of PML. However, because the lack of PML SUMOylation affects the structure and composition of the NBs (Lallemand-Breitenbach et al., 2001), any changes in RNF168 behavior could not be conclusively attributed to a direct requirement for SUMO chains on PML.

Our finding that RNF168 binds hybrid SUMO–ubiquitin chains is also likely to be relevant for RNF168 recruitment to sites of DNA damage. It has been previously reported that SUMO1, SUMO2 and SUMO3 accumulate at double-stranded DNA breaks due to the action of PIAS4 and PIAS1, and that PIAS1 and/or PIAS4 are needed for the productive recruitment of RNF168 (Galanty et al., 2009). We now suggest that this requirement is due to the generation of hybrid SUMO–ubiquitin chains, which are then recognized by RNF168. This would be similar to the recruitment of RAP80 to double-stranded DNA breaks, which has been shown to involve binding to SUMO–ubiquitin hybrid chains generated by RNF4 (Hu et al., 2012; Guzzo et al., 2012).

Our studies have uncovered previously unrecognized roles for the RNF8 and RNF168 DDR proteins in PML regulation. A relationship between DNA repair and PML NBs is well established, in that PML NBs are known to contribute to efficient DNA repair and have been shown to be associated with several DDR proteins (Bischof et al., 2001; Tikoo et al., 2013; Boichuk et al., 2011; Bøe et al., 2006; Yeung et al., 2012; Zhong et al., 2000; Dellaire et al., 2006; Dellaire and Bazett-Jones, 2004). For example, BLM localizes to PML NBs in cells with and without induced DNA damage, and PML is required for the formation of BLM-containing repair foci as well as for BLM function in DNA repair (Zhong et al., 2000; Bischof et al., 2001). However, others report a lack of association of PML with sites of active DNA repair (Dellaire et al., 2006), suggesting that the role of PML in DNA repair is indirect as, for example, in facilitating modifications of DNA-repair proteins (Lallemand-Breitenbach and de The, 2010). Like BLM, we have shown that RNF168 associates with PML NBs and that this interaction also occurs during the DDR. Interestingly, RNF168 is modified by SUMO1 in response to DNA damage (Danielsen et al., 2012) and, because PML NBs can promote SUMOylation, this might be one reason for the association of RNF168 with PML NBs. Whether or not the interaction of RNF168 with PML NBs is necessary for its function in DDR or whether RNF168 has a distinct role in PML regulation remains to be determined.

MATERIALS AND METHODS

Cell lines

The EBV-negative NPC cell line CNE2Z (Sun et al., 1992) was maintained in alpha-MEM (Gibco) supplemented with 10% fetal bovine serum (FBS).

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CNE2Z cell lines expressing single PML isoforms have been described in Sarkari et al. (2011). U2OS cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS.

shRNA screen

CNE2Z cells were seeded onto 384-well glass-bottomed plates (Perkin Elmer 6007550) in 50 µl medium containing 8 µg/ml polybrene at a density of 500 cells/well. 1 h later, 5 µl of shRNA-expressing lentivirus was added, (generated by lentivirus infection of cells in a 96-well plate). The lentivirus library targeted ∼500 ubiquitin pathway proteins with three to five shRNA constructs per gene and is a subset of the library described in Moffat et al. (2006). Infections with each lentivirus were set up in triplicate. 24 h later, 2 µg/ml puromycin was added to select for the virus. The puromycin was removed 24 h later, and the cells were grown for 5 days before fixation and fluorescence staining for image acquisition. Cells were washed with PBS, fixed with 3.7% paraformaldehyde for 25 min, washed with PBS, permeabilized with PBS containing 1% Triton X-100 for 5 min, blocked with 4% BSA in PBS for 2 h and incubated with primary antibody solution (1:100 in 4% BSA in PBS) recognizing PML (Santa Cruz Biotechnology; sc-966) for 18 h at 4°C. The cells were washed with PBS and incubated with blocking buffer containing 1:700 dilution of goat anti-mouse-conjugated Alexa-Fluor-488 (Life Technologies; A11029) and an 1:1000 dilution of 10 mg/ml Hoechst dye 33342 (Molecular Probes). After 1.5 h at room temperature in the dark, the cells were rinsed three times with PBS and stored in PBS. Images from ten areas per well in the green (488) and blue (Hoechst) channels were obtained using a 40× objective on an Opera confocal microscope (PerkinElmer). The number and intensity of PML NBs was determined using Acapella software.

Plasmids and siRNA

Plasmids expressing FLAG-tagged RNF8 (Genecopoeia), RNF168 (pcDNA3:FLAG-RNF168wt) or the RNF168 mutants ΔMIU1 (lacking amino acids 168–191), ΔMIU1 ΔMIU2 (lacking amino acids 168–191 and 439–462), ΔRING (lacking amino acids 1–58), ΔMIU2 (lacking amino acids 439–462) were kindly supplied by Dan Durocher and are described in Stewart et al., 2009. pcDNA3::FLAG ΔUMI ΔMIU1 ΔMIU2 (lacking amino acids 134–191 and 439–462) was generated by PCR amplification of the RNF168 sequences in pGFP-RNF168 ΔUMI ΔMIU1 ΔMIU2 (Panier et al., 2012) and insertion into *Asc*1 and *Xba*1 of pcDNA3:FLAG. Δ100-201ΔMIU2 was generated from pcDNA3::FLAG ΔMIU2 by Quik Change site-directed mutagenesis. pEGFP:NLS-RNF168aa100-201 was obtained from Dan Durocher and is described in Panier et al. (2012). pEGFP:NLS-RNF168 aa100-166 was generated by PCR amplification of the relevant RNF168 sequences, and ligation into *Asc*1 and *Xba*1 sites of pEGFP:NLS. Stealth siRNA of RNF168 (5′-CCAUCCAGCCUCAUCUGGACCAGUU-3′) and RNF8 (5′-GGGUUUGGAGAUAGCCCAAGGAGAA-3′) were from Invitrogen. AllStar negative-control siRNA was obtained from Qiagen.

Antibodies

PML antibodies used for microscopy were from Santa Cruz Biotechnology (sc-966; 1:80 dilution) and those used for western blotting were from Bethyl (A301-167A; 1:2000 dilution). Other antibodies used were mouse anti-FLAG M2 (Sigma; 1:5000 dilution), rabbit anti-FLAG (Bethyl A190-102A; 1:5000 dilution), and antibodies against RNF8 (Santa Cruz 271462; 1:200 dilution), RNF168 (Millipore 06-1130; 1:1000 dilution), HA (Santa Cruz 7392; 1:500 dilution), K63-linked ubiquitin (Millipore 05-1308; 1:1000 dilution), K48-linked ubiquitin (Millipore 05-1307; 1:10000 dilution), Myc (Santa Cruz 40; 1:500 dilution) and actin (Santa Cruz 1616; 1:2000 dilution). Secondary antibodies used for western blots (GAM/HRP-2055, GAR/HRP-2004; 1:5000 dilutions) were from Santa Cruz Biotechnology. Secondary antibodies for microscopy were from Invitrogen (GAM488-A11029, GAM555, GAR488, GAR555; all at 1:700 dilution).

Transfections

Approximately 6×10^5 CNE2Z cells were plated in 5 ml of medium in 10-cm dishes or on coverslips (for microscopy). They were immediately transfected with 100 pmol of siRNA targeted against either RNF8 or RNF168 or AllStars negative control siRNA (Qiagen) using 2 µl of lipofectamine 2000 (Invitrogen). The cells were subject to second and third rounds of the same transfection after 24 h and 48 h, respectively. 48 h after the third round of transfections cells were harvested and either analyzed by western blotting or processed for immunofluorescence microscopy. For overexpression experiments, cells plated as above were transfected with 2 µg of the indicated plasmid using PolyJet (FroggaBio) then harvested 24 h (for PML NB localization experiments) to 48 h (for PML loss experiments) post transfection.

Immunofluorescence microscopy

Cells grown on coverslips were fixed with 3.7% formaldehyde in PBS for 20 min, rinsed twice in PBS and permeabilized with 1% Triton X-100 in PBS for 5 min. Samples were blocked with 4% bovine serum albumin (BSA) in PBS followed by incubation with primary antibodies as indicated, followed by secondary antibodies. Coverslips were mounted on slides using ProLong Gold antifade medium containing 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen). Images were obtained using the 40× oil objective on a Leica inverted fluorescence microscope and processed using the OpenLAB (ver.X.0) software program. PML was quantified by counting 100 cells per sample and experiments were performed in triplicate. Averages and standard deviations were calculated in Excel. *P* values were determined using two-tailed *t*-tests in Excel.

Western blotting

Cells were lysed in 9 M urea,10 mM Tris pH 6.8 followed by sonication. 50 µg of clarified lysates were loaded onto 10% SDS-PAGE and transferred onto nitrocellulose. Membranes were blocked in 5% non-fat dry milk in PBS-T (PBS with 0.1% Tween) for 1 h, followed by incubation with primary antibody in blocking buffer overnight at room temperature. Membranes were washed three times with PBS-T and then incubated with secondary antibodies conjugated to horseradish peroxidase (Santa Cruz Biotechnology) for 1 h. Membranes were washed three times with PBS-T and signals were detected by enhanced chemiluminescence (ECL) (Perkin Elmer Life and Analytical Sciences).

PML ubiquitylation and SUMOylation assays

For ubiquitylation assays, CNE2Z cells in a 15-cm dish were transfected with 5 µg of plasmid expressing HA-tagged ubiquitin and 5 µg of FLAG-RNF168 expression plasmid or empty control plasmid (pcDNA3) by using Polyjet (FroggaBio Scientific Solutions). 40 h post-transfection, cells were treated with 10 µM MG132 (Sigma) for 10 h (except where indicated). Harvested cell pellets were frozen, and then thawed and boiled in 200 µl of SDS lysis buffer (62.5 mM Tris pH 6.8, 2% SDS, 10% glycerol, 1 mM *N*-ethyl maleimide). Clarified lysates were diluted in 1 ml IP buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% NP-40). Lysates were incubated overnight with PML antibody (Bethyl, A301-167A) and Protein A/G agarose (SantaCruz sc-2003). After washing in IP buffer, immunoprecipitates were eluted in loading buffer (60 mM Tris.HCl pH 6.8, 1% SDS, 100 mM DTT, 5% glycerol) prior to western blotting. SUMOylation assays were performed as above except that the plasmid expressing HA-ubiquitin was replaced by Myc-tagged SUMO2 (MacPherson et al., 2009) and no MG132 treatment was used. A positive control for SUMOylation was also performed by treating pcDNA3- and Myc-tagged SUMO2-transfected cells with 4 µM As_2O_3 for 4 h.

Cell-based assay for interaction of RNF168 with SUMO

Cells from the 293T cell line were transfected with 10 µg of plasmid expressing Myc-tagged SUMO1, SUMO2 or SUMO3 (MacPherson et al., 2009) (kindly supplied by Dr Paul Sadowski) and 10 µg of FLAG-RNF168 expression plasmid or empty control plasmid (pcDNA3). 48 h later, cells were lysed in 50 mM Tris pH 7.4, 150 mM NaCl, 1% TritonX-100, 1 mM N-ethyl maleimide and protease inhibitor cocktail (catalog number P8340, Sigma). 1.7 mg of lysate was incubated with 20 µl of M2 FLAG affinity resin (Sigma A2220) for 3 h and then washed four times with 1 ml of lysis buffer. Proteins were eluted in 2×SDS loading buffer [4% (w/v) SDS, 0.2% (w/v) Bromophenol Blue, 20% (v/v) glycerol, 200 mM DTT] and then analyzed by western blotting.

Generation of SUMO2 and SUMO2–ubiquitin affinity resins

4xSUMO2 (SUMO2 residues 2–92 linked to three copies of SUMO2 residues 12–92) and Ub-4xSUMO2 were expressed and purified as described previously (Tatham et al., 2013). Unanchored K63-linked polyubiquitin chains (K63Ub) and K63-linked polyubiquitin chains conjugated to the N-terminus of 4xSUMO2 (K63Ub-4xSUMO2) were synthesized using recombinant Ubc13 and Ube2v2 as described previously (Branigan et al., 2015). These proteins were coupled to N-hydroxysuccinimide (NHS) resin in 0.2 M $NaHCO_3$, 0.5 M NaCl as per manufacturer's instructions (GE Healthcare). The remaining active groups were blocked with ethanolamine, and resins were stored at 4°C.

Identification of cellular proteins that have affinity for SUMO2 and ubiquitin polymers

Nuclear extracts were generated from 293 N3S cells as previously described for U2OS cells (Seifert et al., 2015). Nuclear cell lysate (10 ml at 5.5 mg/ml) was incubated with 50 µl of the affinity resin overnight at 4°C while rotating, then resins were washed three times with RIPA buffer. Bound proteins were eluted by sequential incubation of the resins with 5 µM ubiquitin carboxyl-terminal hydrolase 2 (USP2) and 5 µM of the sentrin-specific protease 1 (SENP1) for 2 h at 22°C, followed by a 5-min incubation at 70°C in denaturing buffer (Novex NuPAGE LDS sample buffer; Life Technologies). SENP1 (415–644) was prepared as described previously (Shen et al., 2006a). 6His-USP2 (259–605) was purified as described for RNF168 below. Since RNF168 was most abundant in the denaturing elutions, only these elutions were used in further analyses. Eluates were fractionated on a NuPAGE Novex 10% Bis-Tris gel run in MOPS buffer, followed by Coomassie-Blue staining. Each lane was cut into upper and lower parts, followed by peptide extraction and in-gel tryptic digestion (Shevchenko et al., 2007). Extracted peptides were resuspended in 35 µl 0.5% acetic acid, 0.1% TFA and 8 µl was analyzed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) using an Q Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific). Data were analyzed using MaxQuant incorporating the Andromeda search engine (version 1.5.2.8) as in Tatham et al. (2011). Protein intensity values based on extracted ion chromatograms (XICs) were reported for each purification. Protein intensities were arithmetically converted to ratio values and ratios were normalized to the median ratio of the entire group to allow for sample-loading errors. Ratio scores were calculated for each protein by comparing data from each affinity resin to the blank resin. Only 69 proteins of the 1827 proteins processed had a ratio score >12 in at least one of the four comparisons. Hierarchical clustering was used to combine the four ratio scores of these proteins into a single heat map by using Perseus.

Assay of purified RNF168 binding to SUMO2 and/or ubiquitin polymers

6His-RNF168 (full length) was expressed in *E.coli* and purified using Ni^{2+}-affinity chromatography, followed by removal of the 6His-tag by tobacco etch virus (TEV) protease digestion and re-purification of the untagged product as previously described (Branigan et al., 2015). 10 µM purified RNF168 was incubated with 20 µl of each affinity resin in 100 µl of binding buffer (50 mM Tris, 150 mM NaCl, 0.5 mM TCEP, 0.05% NP-40) overnight at 4°C. Resins were collected by centrifugation at 500 ***g*** for 1 min, then washed twice with binding buffer. Bound proteins were eluted following sequential incubation with USP2, SENP1 and denaturing buffer as described above. Proteins from the denaturing elution were analyzed by SDS-PAGE and Coomassie-Blue staining.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

L.F., K.S. and R.T.H. conceived of and designed the experiments. K.S., A.I.W., M.H.T., O.F.A., D.R. and S.G. performed the experiments. J.M. provided the lentivirus shRNA library and the microscope for the initial screen. L.F. wrote the manuscript.

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Supplementary information

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