Sequential ubiquitination and deubiquitination enzymes synchronize the dual sensor and effector functions of TRIM21

Adam J. Fletcher[1], Donna L. Mallery, Ruth E. Watkinson, Claire F. Dickson, and Leo C. James[1]

Division of Protein and Nucleic Acid Chemistry, Medical Research Council Laboratory of Molecular Biology, Cambridge CB2 0QH, United Kingdom

Edited by Jeremy Luban, University of Massachusetts Medical School, Worcester, Massachusetts, and accepted by the Editorial Board June 15, 2015 (received for review April 17, 2015)

Tripartite motif (TRIM) 21 is a cytosolic antibody receptor that neutralizes antibody-coated viruses that penetrate the cell and simultaneously activates innate immunity. Here we show that the conjugation of TRIM21 with K63-linked ubiquitin (Ub-^{63}Ub) catalyzed by the sequential activity of nonredundant E2 Ub enzymes is required for its dual antiviral functions. TRIM21 is first labeled with monoubiquitin (monoUb) by the E2 Ube2W. The monoUb is a substrate for the heterodimeric E2 Ube2N/Ube2V2, resulting in TRIM21-anchored Ub-^{63}Ub. Depletion of either E2 abolishes Ub-^{63}Ub and Ub-^{48}Ub conjugation of TRIM21, NF-κB signaling, and virus neutralization. The formation of TRIM21-Ub-^{63}Ub precedes proteasome recruitment, and we identify an essential role for the 19S-resident and degradation-coupled deubiquitinase Poh1 in TRIM21 neutralization, signaling, and cytokine induction. This study elucidates a complex mechanism of step-wise ubiquitination and deubiquitination activities that allows contemporaneous innate immune signaling and neutralization by TRIM21.

TRIM21 | NF-kB | Ube2W | Ube2N/Ube2V2 | Poh1

TRIM21 is a cytoplasmic Ig receptor belonging to the tripartite motif family of E3 ubiquitin (Ub) ligases. Many TRIM proteins (there are ∼100 members in humans) have reported roles in innate immunity (1). TRIM21 intercepts incoming, cytoplasmic antibody-coated pathogens and neutralizes them by mediating their VCP- and proteasome-dependent degradation (2, 3). In addition, TRIM21 initiates a signaling cascade, resulting in the transcriptional up-regulation of inflammatory cytokines and a potent antiviral state in surrounding cells (4). Such antiviral duality has also been reported for a paralogue of TRIM21, TRIM5α (5), and more recently, TRIM19/PML (6), suggesting that an ability to "sense" and neutralize viral infection is a conserved TRIM function. The mechanism by which a TRIM protein coordinates sensor and effector functions remains unknown.

For TRIM21, these dual functions are dependent upon its activity as an E3 Ub ligase. In previous work, we have shown that the TRIM21 really interesting new gene (RING) domain is required for neutralization and catalyzes autoubiquitination with K48-linked polyubiquitin (polyUb) (Ub-^{48}Ub, following the nomenclature suggested by ref. 7) in vitro (3), suggesting TRIM21 might synthesize Ub-^{48}Ub to recruit the proteasome. We also demonstrated that TRIM21, with the heterodimeric E2 enzyme Ube2N/Ube2V1, catalyses the formation of unanchored K63-linked polyUb (Ub-^{63}Ub) in vitro (4). Ube2N and Ub-^{63}Ub were essential for TRIM21 to mount an innate signaling response, consistent with a role for unanchored Ub-^{63}Ub in NF-κB signaling (8). Thus, the ability of TRIM21 to mediate multiple functions seems associated with its ability to synthesize multiple Ub chain types. Moreover, because TRIM21 only initiates innate signaling upon recognition of antibody-bound virus (4), this suggests that the effector and sensor functions of TRIM21 are mechanistically connected.

Four enzymes are involved in the ubiquitination cycle: the Ub activating (E1), the Ub conjugating (E2), the substrate-specifying Ub ligase (E3), and the Ub-erasing deubiquitinase (DUB). Of these, the E2 plays a central role in selecting the Ub chain linkage topology (9–11). For example, the heterodimeric RING-domain E3 ligase BRCA1-BARD1 auto-monoubiquitinates using one of the E2s UbcH6, Ube2E2, UbcM2, or Ube2W, and the monoUb can be extended into Ub-^{63}Ub or Ub-^{48}Ub by Ube2N/UbeV2 or Ube2K, respectively (12). PolyUb is typically attached to a substrate by the E3; however, with some proclivity, E3–E2 pairs can also generate unanchored polyUb chains in vitro (13). Unanchored polyUb has been shown to activate innate immune signaling (4, 5, 8, 14), but how it is generated and regulated in cells remains poorly understood.

Once a Ub-modified substrate arrives at the proteasome, three DUBs in the 19S regulatory particle (RP), Usp14/Ubp6, Uch37/UCHL5, and Rpn11/Poh1, trim or remove Ub modifications before substrate degradation (15). Although Ub-^{48}Ub is a canonical degradation signal, both Ub-^{48}Ub and Ub-^{63}Ub are hydrolyzed by purified proteasomes in vitro (16, 17), and some studies have observed Ub-^{63}Ub specificity in the polyUb amputation activity of Poh1 (18, 19). Whether Ub-^{63}Ub has a role at the proteasome remains unclear; its presence at this location would seem incongruous with a model in which only Ub-^{48}Ub-labeled proteins are genuine substrates (20, 21).

In this report, we show that the effector and sensor functions of TRIM21 are dependent on its sequential recruitment of the E2s Ube2W and Ube2N/Ube2V2, and the DUB Poh1.

Significance

Tripartite motif (TRIM) proteins are a large family of E3 ubiquitin (Ub) ligases, with many members having important roles in innate immunity. TRIM21 is a cytosolic antibody receptor that recognizes the Fc portion of antibodies bound to incoming virions. On binding to these immune complexes, TRIM21 triggers the catastrophic disassembly of viral capsids at the proteasome, terminating viral infection. Simultaneously, TRIM21 "senses" the presence of the virus and provokes signaling cascades that activate the transcription factor NF-κB, ultimately alerting surrounding cells to the infection. Here, we uncover the stepwise ubiquitination mechanism catalyzed by TRIM21, as well as the various cofactors required, that allows these two antiviral activities to occur synchronously at the proteasome.

Author contributions: A.J.F., D.L.M., R.E.W., and L.C.J. designed research; A.J.F., D.L.M., and R.E.W. performed research; A.J.F., D.L.M., R.E.W., and C.F.D. contributed new reagents/analytic tools; A.J.F., D.L.M., R.E.W., C.F.D., and L.C.J. analyzed data; and A.J.F. and L.C.J. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. J.L. is a Guest Editor invited by the Editorial Board.

See Commentary on page 9797.

[1]To whom correspondence may be addressed. Email: fletcher@mrc-lmb.cam.ac.uk or lcj@mrc-lmb.cam.ac.uk.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1507534112/-/DCSupplemental.

Results

Ube2W and Ube2N Are Required for TRIM21 Dual Effector and Sensor Functions. Previously, we have shown that infection of nonprofessional cells with antibody-coated adenovirus (AdV:Ab) results in TRIM21-dependent NF-κB activation and proteasome-mediated virus neutralization (4). To investigate the functional relationship between these two antiviral outcomes, we first asked whether the generation of an innate immune response via NF-κB activation precedes virus destruction by the proteasome. We treated human TE671 cells with chemical inhibitors of the proteasome (MG132, bortezomib) or NF-κB signaling (5Z-7-Oxozeaenol, IKK Inhibitor VII). As shown in Fig. 1*A*, 7-h treatment of human TE671 cells with MG132, bortezomib, IKK VII, or 5Z-7-Oxozeaenol inhibited the stimulation of a model NF-κB promoter that drives luciferase expression (NF-κB-Luc) after infection with AdV:Ab. However, although both MG132 and bortezomib inhibited virus neutralization by the monoclonal antihexon antibody 9C12 (Fig. 1*B*), IKK inhibitor VII or 5Z-7-Oxozeaenol did not. Thus, TRIM21-dependent NF-κB activation is not a requisite for virus neutralization, suggesting signaling and neutralization might be synchronous, rather than sequential.

To test this idea, we considered whether TRIM21 ubiquitination, as required for innate signaling, was also required for virus neutralization. In vitro, TRIM21 synthesizes unanchored Ub-^{63}Ub in the presence of Ube2N/Ube2V1, and both Ub-^{63}Ub and Ube2N are necessary for TRIM21 to provoke an innate immune signal (4). In Ube2N-depleted cells, 9C12-mediated neutralization of AdV-GFP was largely diminished (Fig. 1*C*), suggesting the formation of Ub-^{63}Ub precedes viral neutralization. However, proteins are typically targeted with anchored Ub chains to recruit the proteasome. Because Ube2W is described to facilitate the Ube2N/Ube2V2-mediated polyubiquitination of substrates with anchored Ub-^{63}Ub chains (12, 22), we depleted Ube2W, using shRNA, to ask whether it too has a role in virus neutralization. Interestingly, Ube2W depletion also reversed neutralization of adenovirus by 9C12 (Fig. 1*C*), suggesting both Ube2W and Ube2N/Ube2V2 are cofactors for TRIM21-mediated virus neutralization. Furthermore, we observed that Ube2W depletion abolished potent activation of NF-κB-Luc on challenge with Adv:Ab (Fig. 1*D*). In contrast, we measured no decrease in NF-κB-Luc activation in response to TNFα in Ube2W-depleted cells (Fig. 1*E*). Western blots confirmed depletions of Ube2N (Fig. 1*F*), and RT-qPCR confirmed depletions of Ube2W (Fig. 1*G*). Together, these experiments suggest Ube2N and Ube2W are nonredundant cofactors for both sensing and effector responses of TRIM21, an unexpected observation because Ube2W is reported to anchor polyUb to substrates (22, 23) and Ub-^{63}Ub chains are not thought to recruit proteins to the proteasome (17).

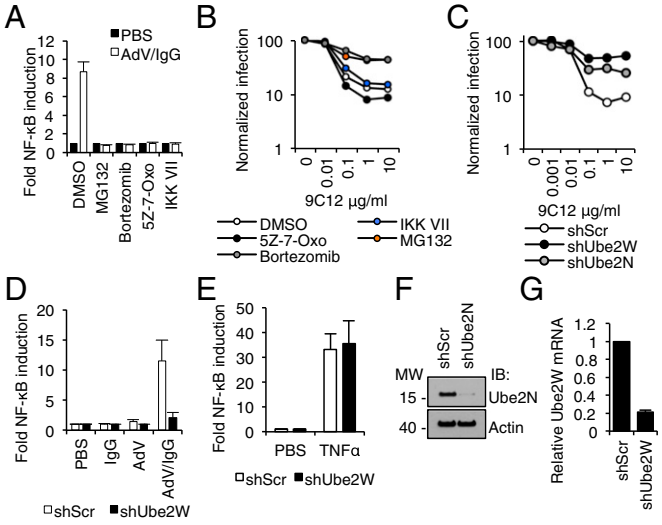

Fig. 1. Ube2W and Ube2N are required for TRIM21-mediated signaling and neutralization. (*A*) Proteasome or NF-κB signaling inhibitors prevent NF-κB activation in response to AdV/IgG. (*B* and *C*) TE671 cells, infected with AdV (MOI ~0.1), preincubated with 9C12 or PBS, treated with the inhibitors indicated or DMSO (*B*), or expressing scrambled shRNA (shScr) (*C*–*G*), Ube2N-specific shRNA (shUbe2N) (*C* and *F*) or Ube2W-specific shRNA (shUbe2W) (*C*–*E* and *G*), percentage infection quantified by flow cytometry for GFP expression 24 h postinfection, values normalized to PBS-treated AdV control. (*D* and *E*) TE671-NF-κB-Luc cells expressing shScr or shUbe2W challenged with PBS (*D* and *E*), human IgG, AdV, AdV/IgG (*D*), or human TNFα (*E*) for 7 h. (*F*) Immunoblot of TE671-shScr or TE671-shUbe2N, detecting Ube2N or β-actin (loading control). (*G*) Quantitative RT-PCR detecting Ube2W mRNA in TE671-shScr or TE671-shUbe2W. Values calculated using the ΔΔCt method and normalized to β-Actin mRNA. All error bars are SEM of duplicates or triplicates.

Ube2W Monoubiquitinates TRIM21. We next investigated whether TRIM21 can act as an E3 enzyme for Ube2W. We expressed full-length TRIM21 bearing an N-terminal MBP tag that improved protein solubility. We incubated purified MBP-TRIM21 with E1, Ube2W, and Ub. As increasing amounts of Ube2W were titrated into the reactions, we observed an increase in modified MBP-TRIM21 (Fig. 2*A*, *Top*). Probing with an anti-Ub antibody revealed a band of the same size as modified MBP-TRIM21, suggesting the adduct was a monoUb (Fig. 2*A*, *Bottom*). There were additional bands in the Ub blot with sizes coincident with mono- and diubiquitinated Ube2W (Fig. 2*A*, *Bottom*) (22).

To support our interpretation that TRIM21 undergoes auto-monoubiquitination using Ube2W, we purified untagged TRIM21 corresponding to the RING and B-Box2 domains (residues 1–129) only, and incubated purified TRIM21$^{\text{RING-Box}}$ with E1, Ube2W, and Ub. As Ube2W was titrated into these reactions, we observed the pronounced appearance of a band corresponding to modified TRIM21$^{\text{RING-Box}}$ (Fig. 2*B*, *Top*). These reactions were driven to near-completion, with the appearance of modified TRIM21$^{\text{RING-Box}}$ correlating with a decrease in the levels of unmodified TRIM21$^{\text{RING-Box}}$ (Fig. 2*B*, *Top*). Probing the same blots with an anti-Ub antibody confirmed the modified TRIM21$^{\text{RING-Box}}$ band to be monoubiquitinated TRIM21$^{\text{RING-Box}}$ (Fig. 2*B*, *Bottom*). As in the previous reactions, we found that Ube2W was also mono- and diubiquitinated (Fig. 2*B*, *Bottom*). These experiments demonstrate that TRIM21 is an E3 ligase capable of auto-monoubiquitination with the E2 Ube2W in vitro.

Monoubiquitinated TRIM21 Is a Substrate for Ube2N/Ube2V2. Previously we have shown in vitro that in the presence of E1, Ub, and Ube2N TRIM21 can catalyze the formation of unanchored Ub-^{63}Ub (4). We next investigated whether this pattern of activity is altered in the presence of Ube2W. Similar to previous experiments with full-length protein, incubation of TRIM21$^{\text{RING-Box}}$ with Ube2N/Ube2V2 resulted in the production of Ub chains that were not conjugated to TRIM21 (Fig. 2*C*). However, in the presence of Ube2W, polyubiquitination of TRIM21$^{\text{RING-Box}}$ was observed (Fig. 2*D*). This was accompanied by an overall increase in the levels of polyubiquitin chains, as detected by an anti-Ub antibody, suggesting Ube2W also potentiates the reaction between Ube2N/Ube2V2 and TRIM21 (Fig. 2*D*, *Bottom*). Extension of monoubiquitinated TRIM21$^{\text{RING-Box}}$ by Ube2N/Ube2V2 was dose-dependent, as increasing Ube2N/Ube2V2 concentrations increased the length of the conjugated Ub chain (Fig. 2*D*, *Top*).

Repeating the reaction with intermediate E2 concentrations and various Ub mutants confirmed that, as expected for Ube2N/Ube2V2, polyubiquitination of TRIM21$^{\text{RING-Box}}$ occurred through a K63 linkage (Fig. 2*E*). K63R mutant Ub was not conjugated to monoubiquitinated TRIM21 (Fig. 2*E*, lane 3).

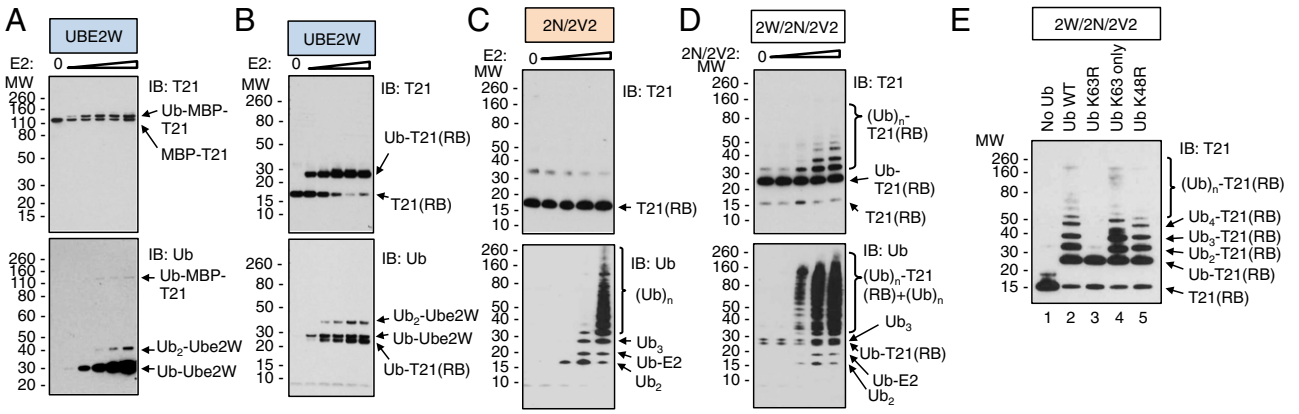

Fig. 2. TRIM21 autoubiquitinates with Ube2W and Ube2N/Ube2V2 in vitro. (*A* and *B*) Ube2W monoubiquitinates TRIM21 (T21). Purified MBP-T21 (*A*) or T21$^{RING\text{-}Box}$ (RB) (*B*), E1, Ub, and ATP were incubated with increasing concentrations of Ube2W. Reaction products separated by LDS-PAGE and visualized by IB detecting T21 (*Top*) or Ub (*Bottom*). (*C* and *D*) Ube2W anchors polyUb to T21. Ubiquitination reactions as in *B*, but with increasing concentrations of Ube2N/Ube2V2 (2N/2V2) in the absence (*C*) or presence (*D*) of Ube2W (2W). (*E*) T21(RB) autoubiquitinates with Ub-^{63}Ub. T21(RB) incubated with E1, ATP, 2W, 2N/2V2, and WT Ub or Ub bearing lysine-to-arginine substitutions at residue 63 (K63R), residue 48 (K48R), or at all Ub lysine residues except residue 63 (K63 only).

Conversely, both a mutant Ub bearing only one lysine at residue 63 (Fig. 2*E*, lane 4) and mutant Ub bearing arginine in place of lysine 48 (Fig. 2*E*, lane 5) were as efficiently incorporated into TRIM21-linked polyubiquitin as WT Ub (Fig. 2*E*, lane 2), confirming K63-linkage homogeneity of these chains.

Ube2W and Ube2N/Ube2V2 Cooperatively Polyubiquitinate a Lysine-Less TRIM21$^{RING\text{-}Box}$. It has recently emerged that Ube2W does not require lysines for Ub conjugation and preferentially transfers Ub to the primary N-terminal α-amino group of several proteins in vitro, including Ube2W itself (22–24). To assess whether TRIM21 is also ubiquitinated by Ube2W independent of lysine residues in vitro, we purified TRIM21$^{RING\text{-}Box}$ proteins bearing lysine-to-arginine substitutions at all six lysine residues in TRIM21$^{RING\text{-}Box}$: three in the RING domain (Lys45, Lys61, Lys77) and three in the B-Box2 domain (Lys105, Lys108, Lys119). TRIM21$^{RING\text{-}Box}$-6KR (K45/61/77/105/108/119R) was purified and incubated with E1, Ube2W, and Ub, with or without ATP. Coomassie Blue-stained LDS-PAGE revealed that the mutant protein was fully active with Ube2W, as it was converted into a modified TRIM21$^{RING\text{-}Box}$ species as efficiently as WT TRIM21$^{RING\text{-}Box}$ (Fig. 3*A*, lanes 2 and 4). Mutation of the lysine residues also had no effect on Ub ligase activity, as TRIM21$^{RING\text{-}Box}$-6KR was able to generate free Ub-^{63}Ub in the presence of Ube2N/Ube2V2 (Fig. 3*B*, lanes 2 and 4). The addition of Ube2W to the Ube2N/Ube2V2-containing reactions resulted in a change in high molecular weight (HMW) reaction products, consistent with TRIM21$^{RING\text{-}Box}$-6KR auto-polyubiquitination (Fig. 3*C*). To confirm this interpretation, we incubated increasing amounts of WT or 6KR mutant TRIM21$^{RING\text{-}Box}$ with either Ube2W or Ube2W and Ube2N/Ube2V2, and probed immunoblots with a TRIM21 antibody. Both TRIM21 variants were efficiently modified in the presence of Ube2W (Fig. 3 *D* and *E*, *Left*). The addition of Ube2N/Ube2V2 to these reactions led to the polyubiquitination of TRIM21 (Fig. 3 *D* and *E*, *Right*). Thus, lysine residues are not required for ubiquitination of TRIM21$^{RING\text{-}Box}$ by Ube2W and Ube2N/Ube2V2 in vitro, in agreement with the N terminus of TRIM21 being a potential target for ubiquitination by Ube2W.

Ube2W and Ube2N Are Necessary for Ubiquitination of TRIM21 in Cells. We next asked whether the requirement for Ube2W and Ube2N during TRIM21-mediated neutralization of virus infection (Fig. 1) correlates with a dependence on these enzymes for ubiquitination of TRIM21 in cells. To test this, we sought evidence for ubiquitinated TRIM21 by expressing TRIM21 with an N-terminal 6xHis-tag, together with HA-tagged Ub in control TE671 cells or cells depleted of either Ube2W or Ube2N. Cell lysate was incubated with Ni-NTA beads and captured protein blotted for total Ub. A strong, HMW polyubiquitinated smear was observed in control cells expressing 6xHis-TRIM21 and HA-Ub but was absent in cells that were depleted of Ube2N (Fig. 4*A*) or Ube2W (Fig. 4*B*), suggesting TRIM21 is ubiquitinated in cells by these two E2s. We next probed purified proteins from control or Ube2N-depleted lysates with antibodies specific for either

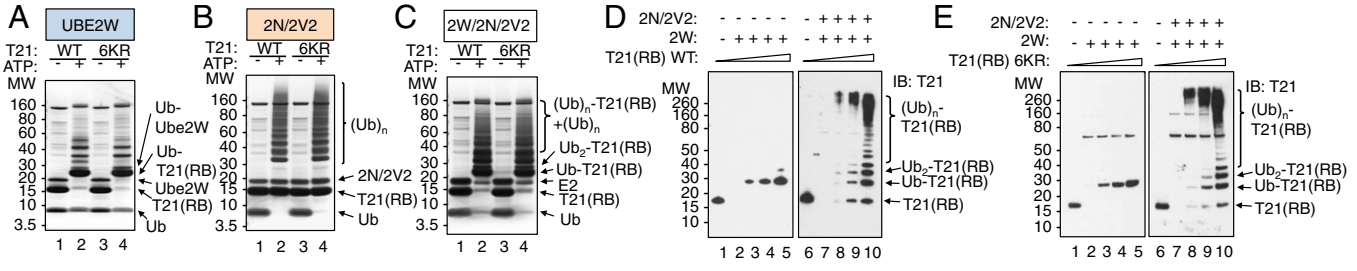

Fig. 3. A lysine-less TRIM21$^{RING\text{-}Box}$ autoubiquitinates with Ube2W and Ube2N/Ube2V2 in vitro. (*A*–*C*) WT or mutant TRIM21$^{RING\text{-}Box}$-6KR proteins bearing lysine-to-arginine at residues 45, 61, 77, 105, 108, and 119 were incubated with E1 and Ub in the presence or absence of ATP and Ube2W (*A*), Ube2N/Ube2V2 (*B*), or Ube2W and Ube2N/Ube2V2 (*C*). Reactions products resolved by LDS-PAGE and visualized by Coomassie stain. (*D* and *E*) Autoubiquitination of WT (*D*) or 6KR (*E*) TRIM21$^{RING\text{-}Box}$ in the presence of Ube2W (*Left*) or Ube2W and Ube2N/Ube2V2 (*Right*). Reaction products resolved by LDS-PAGE and visualized by immunoblot detecting TRIM21. Reactions containing TRIM21$^{RING\text{-}Box}$ in the absence of any E2 served as controls.

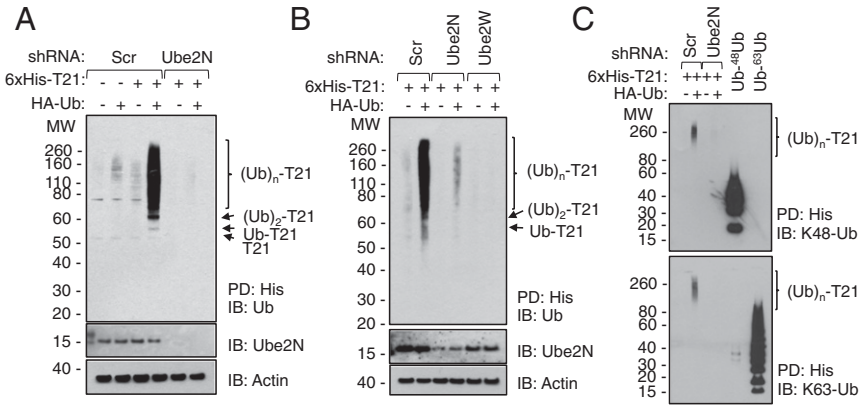

Fig. 4. K48- and K63-linked ubiquitination of cellular TRIM21 requires Ube2W and Ube2N. (*A*–*C*) TE671 expressing a scrambled shRNA (Scr), Ube2N-specific shRNA, or Ube2W-specific shRNA (*B*) and the indicated combinations of 6xHis-TRIM21 and HA-Ub. Cell lysates were incubated with Ni^{2+} affinity resin, and input and pull-down (PD) fractions were resolved by LDS-PAGE and detected by IB with antibodies against total Ub, Ube2N, Ub-^{48}Ub, Ub-^{63}Ub, or β-Actin (loading control). In *C*, 1 μg purified Ub-^{48}Ub or Ub-^{63}Ub was loaded to control for antibody specificity.

K48 or K63 isopeptide bonds. To control for antibody linkage specificity, we loaded recombinant polyubiquitin linked by either K48 or K63 linkages. Importantly, we observed robust labeling of TRIM21 by both K48- and K63-linked polyUb in control cells, but not in cells depleted of Ube2N (Fig. 4*C*).

Taken together, the data so far fit a model in which TRIM21 is first monoubiquitinated by Ube2W and then polyubiquitinated by Ube2N/Ube2V2. Given the requirement for each of these E2s in the dual effector–sensor functions of TRIM21 (Fig. 1) and for Ub-^{48}Ub and Ub-^{63}Ub labeling in cells (Fig. 4), we hypothesized that polyubiquitination of TRIM21 is a critical initial step in TRIM21 antiviral function.

The Proteasome-Associated Deubiquitinase Poh1 Is Required for TRIM21 Signaling. We next considered how TRIM21-anchored Ub-^{63}Ub could activate immune signaling. We hypothesized that anchored Ub-^{63}Ub could be liberated during proteolysis at the proteasome, enabling its binding to downstream signaling effectors such as TAB2 (5, 8). Such a mechanism would allow stimulation of innate signaling to occur synchronously with neutralization of virus, rationalizing the requirement for Ube2W in both functions (Fig. 1). Of the three DUBs associated with the proteasome 19S RP, Poh1 uniquely cleaves an isopeptide bond between the proximal Ub in a polyUb adduct and its substrate, thereby releasing a polyUb chain en bloc (16, 25, 26). To test the notion that Poh1 might deubiquitinate TRIM21, we stably depleted Poh1 in TE671 cells by shRNA expression, assessing efficiency by immunoblot (Fig. 5*A*). Specific purification of 6xHis-TRIM21 from control or Poh1-depleted cells revealed increased Ub-^{63}Ub conjugation to TRIM21 after Poh1 depletion (Fig. 5*B*). To determine whether the persistence of Ub-^{63}Ub on TRIM21 bore a consequence for immune signal induction, we infected control or Poh1-depleted cells with AdV:Ab and measured stimulation of NF-κB-Luc. Whereas in control cells, AdV:Ab robustly stimulated luciferase expression, cells depleted of Poh1 were largely unresponsive to challenge (Fig. 5*C*), supporting a role for Poh1 in TRIM21 innate signal induction.

Protein levels of the 20S core α1–7 subunits (Fig. S1*A*) or the 19S RP-associated Ub acceptor Rpn13/Adrm1 (Fig. S1*A*) were not diminished in Poh1-depleted cells. Moreover, Poh1 depletion did not alter the degradation of the model proteasome degradation substrate Ub-G76V-GFP (27); Poh1 depletion neither caused accumulation of Ub-G76V-GFP in DMSO-treated cells nor affected the magnitude of stabilization by MG132 (Fig. 5*D*). Together, this suggests stable Poh1 depletion does not nonspecifically disrupt proteasomes. However, the ability of TRIM21 to facilitate 9C12-mediated virus neutralization was diminished in cells depleted of Poh1 (Fig. 5*E*), suggesting Poh1 is a necessary cofactor for both of TRIM21's antiviral functions. We repeated this experiment in mouse embryonic fibroblasts (MEFs), using the mouse monoclonal 9C12, and observed a similar substantial reduction in Adv neutralization, suggesting Poh1 requirement is conserved across mammalian species (Fig. 5*F*).

To further substantiate the requirement for Poh1, we assessed its importance during inflammatory cytokine induction by TRIM21. We infected WT and TRIM21-knockout (K21) MEFs with AdV:Ab and determined immune activation by measuring TNFα transcription. As previously observed (4), potent up-regulation of TNFα transcription by AdV:Ab in WT MEFs was largely abolished in K21 MEFs (Fig. 5*G*), confirming the transcriptional response to be TRIM21-dependent. We transiently depleted Poh1 from these cells by siRNA, measuring Poh1 transcript expression by qPCR (Fig. 5*H*). In WT MEFs, the up-regulation of TNFα transcription by AdV:Ab was significantly reduced by Poh1 depletion (Fig. 5*I*). Conversely, the minimal TRIM21-independent TNFα transcription observed in K21 cells was Poh1-independent (Fig. 5*J*), suggesting NF-κB activation per se was not inhibited by Poh1 depletion. In agreement with this observation, the ability of the RIG-I agonist poly(I:C) to trigger expression of NF-κB-dependent genes was unaffected by Poh1 depletion (Fig. 5*K* and Fig. S1 *B* and *C*), supporting the notion that Poh1 depletion does not inhibit signaling nonspecifically. Poh1 was also required for cytokine transcription in human cells infected with AdV:Ab. Depletion of Poh1 in HeLa cells (Fig. 5*L*) significantly repressed the up-regulation of IL-8 (Fig. 5*M*) and IL-6 (Fig. 5*N*) transcription on AdV:Ab challenge. As in MEFs, Poh1 depletion had no effect on NF-κB stimulation by the RIG-I agonist poly(I:C) (Fig. 5*N*). Together, these data suggest Poh1 has a specific role in TRIM21-mediated signaling during infection by antibody-coated virus.

In performing these experiments, we were cautious of the fact that the polyubiquitination and proteasome-mediated degradation of IκBα after its phosphorylation by IKKβ is required for NF-κB nuclear translocation (28). To further control for nonspecific effects of Poh1 depletion on NF-κB signaling, we cotransfected IKKβ and NF-κB-Luc into control 293T cells or cells transiently depleted of Poh1, assessing depletions by immunoblot (Fig. S1*D*). In parallel, we treated 293T cells with vehicle (DMSO) or bortezomib for the duration of the experiment. Transfection of IKKβ potently stimulated NF-κB-Luc activation in both control and Poh1-depleted cells (Fig. S1*E*). However, proteasome inhibition by bortezomib abolished all signaling activity (Fig. S1*E*), suggesting Poh1 depletion does not inhibit NF-κB signaling through proteasome inhibition (Fig. S2).

Discussion

In this study, we find that TRIM21 sensor and effector functions both derive from a TRIM21-anchored Ub-^{63}Ub chain, synthesized in conjunction with the E2 Ub conjugating enzymes

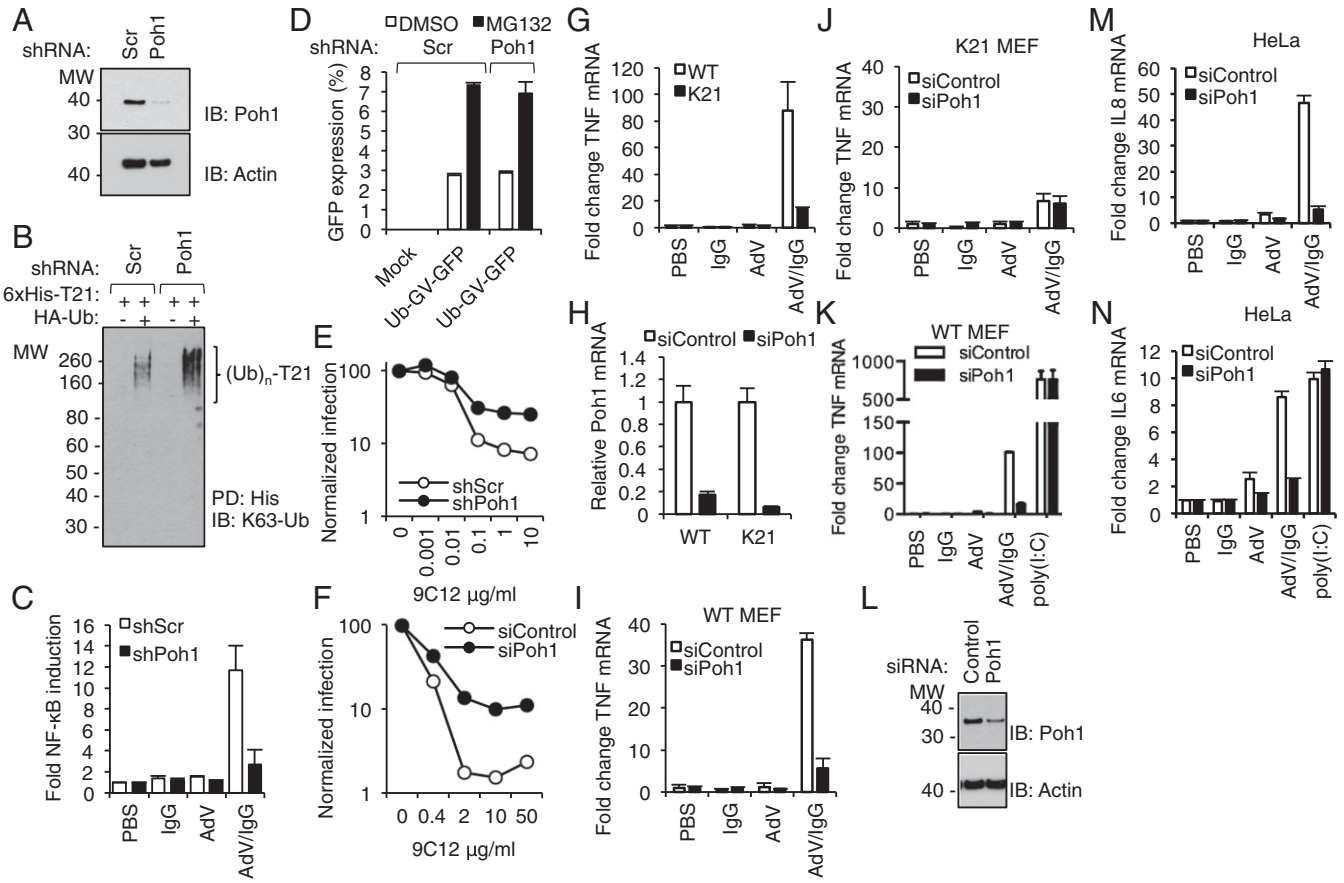

Fig. 5. Poh1-mediated NF-κB activation and cytokine transcription in response to AdV/IgG in human and mouse cells. (*A*) IB detecting Poh1 or β-Actin (loading control) in TE671 expressing a scrambled shRNA (shScr) or Poh1-specific shRNA (shPoh1). (*B*) Pull-down of 6xHis-TRIM21 (T21) from TE671 expressing shScr or shPoh1, and precipitated proteins assessed by IB detecting Ub-^{63}Ub. (*C*) TE671-NF-κB-Luc cells expressing shScr or shPoh1 incubated with PBS, AdV, IgG, or AdV/IgG. NF-κB activation determined by luminometry, values expressed relative to PBS control in each condition. (*D*) TE671 expressing shScr or shPoh1 and Ub-GV-GFP, treated with MG132 for 10 h, GFP-positive cells enumerated by flow cytometry. (*E*) TE671 expressing shScr or shPoh1 infected with AdV (MOI ~0.2) preincubated with increasing concentrations of 9C12. Percentage infection determined by flow cytometry detecting GFP. Values normalized to infection in the presence of PBS alone. (*F*) WT MEFs transfected with nonspecific siRNA (siControl) or Poh1-specific siRNA (siPoh1) and infected as in *E*. (*G*) WT or TRIM21$^{-/-}$ (K21) MEFs incubated with PBS, IgG, AdV, or AdV/IgG. Quantitative RT-PCR (qRT-PCR) was performed, and TNFα mRNA copies relative to TBP mRNA copies were quantified 4 h postchallenge. (*H*) qRT-PCR measuring Poh1 mRNA copies relative to TBP mRNA copies in WT or K21 MEFs transfected with siControl or siPoh1. (*I–K*) WT (*I*, *K*) or K21 (*J*) MEFs transfected with siControl or siPoh1, incubated with PBS, IgG, AdV, AdV/IgG (*I–K*), or poly(I:C) (*K*). qRT-PCR performed and TNFα mRNA copies relative to TBP mRNA copies quantified 4 h postchallenge. (*L*) IB detecting Poh1 or β-Actin (loading control) in HeLa transfected with siControl or siPoh1. (*M* and *N*) HeLa transfected with siControl or siPoh1 incubated with PBS, IgG, AdV, AdV/IgG (*M* and *N*) or poly(I:C) (*N*). qRT-PCR performed and IL-8 (*M*) or IL-6 (*N*) mRNAs relative to β-Actin mRNA copies quantified 4 h postinfection. Values are means of triplicates ± SEM.

Ube2W and Ube2N/Ube2V2. These results are in good agreement with previous reports of cooperation between Ube2W and Ube2N/Ube2V2 (12, 22) and the ability of Ube2W to mediate specific N-terminal ubiquitination of substrates (22–24). In the absence of Ube2W, Ube2N/Ube2V2 and TRIM21 synthesize unanchored Ub-^{63}Ub in vitro. This reaction looks sufficient to generate the polyUb stimulus of NF-κB signaling because unanchored Ub-^{63}Ub is alone able to activate TAK1 (8). Indeed, it is possible that unanchored polyUb is generated in cells via the same mechanism as it is generated in vitro. However, our finding that Ube2W is a critical component of TRIM21-mediated immune signaling, when considered alongside its Ub chain-anchoring activity (Figs. 1 and 2) (12, 22–24), suggests immunostimulatory Ub-^{63}Ub chains are first synthesized as adducts of TRIM21. Under this model, TRIM21-anchored Ub-^{63}Ub chains could either bind downstream receptors while anchored to TRIM21 or represent precursors of unanchored Ub-^{63}Ub chains (8).

Our data support a requirement for TRIM21-anchored Ub-^{63}Ub to be liberated before immune activation. Depletion of the 19S DUB Poh1 prevents neutralization and signaling and leads to increased TRIM21 polyubiquitination. Although we have not directly demonstrated that it is Ub-^{63}Ub released by Poh1 that activates NF-κB, such a model is mechanistically attractive. The requirement for Poh1 in both neutralization and signaling could be explained if the release of Ub-^{63}Ub by Poh1 is coupled to translocation into the proteasome, allowing degradation. Ub must be removed from a substrate before translocation into the proteolytic proteasome core particle (15). Poh1 is a degradation-coupled DUB (25, 29), with Ub-^{63}Ub specificity in vitro (18, 19) and in cells (30). Electron microscopy experiments position Poh1's active site directly above the pore formed by the heterohexameric ATPase ring of the 19S base, through which a substrate must pass before entering the core particle (31–33). Although the removal en bloc of a Ub chain by Poh1 is a noted feature of its mechanism (26, 34), and one that distinguishes it from other 19S DUBs (15), the reason for this singular activity is not well understood. Our finding that Poh1 is required for signaling provides a plausible rationale for its liberation of Ub-^{63}Ub.

Our data do not address how the proteasome is recruited by TRIM21. Although we observe that Ube2W and Ube2N synthesize

anchored Ub-^{63}Ub in vitro, depletion of either E2 results in a loss of TRIM21 conjugation with both Ub-^{63}Ub and Ub-^{48}Ub in cells. This may indicate that Ub-^{63}Ub formation precedes Ub-^{48}Ub conjugation and that it is the loss of Ub-^{48}Ub that prevents proteasomal recruitment. However, we have no direct evidence that K48 polyubiquitination is required for virus degradation by TRIM21. Several E2s have been shown to synthesize Ub-^{48}Ub with TRIM21 in vitro (3, 35), suggesting Ub-^{48}Ub-conjugating activity in cells might be redundant. Unraveling the role of Ub-^{48}Ub in TRIM21 function may also be complicated by the fact that downstream pathways induced by TRIM21 may require Ub-^{48}Ub conjugation. For instance, activation of NF-κB requires Ub-^{48}Ub-dependent IκBα degradation (28).

An important question raised by our results is why TRIM21 uses a complex mechanism of sequential ubiquitination in which anchored Ub-^{63}Ub chains are a precursor to proteasome recruitment. One reason may be to ensure that efficient degradation of the viral particle does not antagonize the ability of TRIM21 to trigger innate signaling. Linking TRIM21 function closely with a controlled process of ubiquitination and deubiquitination allows for exquisite regulation, synchronizing signaling with virus degradation, and could limit spurious unanchored Ub-^{63}Ub production as a result of stochastic TRIM21:Ube2N interaction in cells, in the absence of infection. Several TRIM proteins are thought to synthesize Ub-^{63}Ub with immunomodulatory function, including TRIM5α (5) and TRIM25 (36). It will be interesting to consider whether sequential ubiquitination and deubiquitination are general features of TRIM biology, evolved to coordinate sensor and effector antiviral duality.

Materials and Methods

Cells, RNAi. TE671, HeLa, 293T, and MEF cells were maintained in DMEM supplemented with 10% (vol/vol) FCS, penicillin at 100 U/mL, and streptomycin at 100 μg/mL. For RNAi, cells were transfected with Poh1 SMARTpool siRNA or control oligonucleotides, using Oligofectamine or RNAiMAX. Retroviral particles were generated by cotransfection of 293Ts with pCMVi GagPol, pMDG VSVgp, and shRNA-expressing vectors using Fugene-6. DNA constructs, shRNA sequences, antibodies, and inhibitors are listed in *SI Materials and Methods*.

Infection Assays. AdV-GFP incubated with or without antibody for 1 h before addition to cells. Percentage GFP determined by flow cytometry 24–48 h postinfection. For luciferase assays, cells were transfected with pGL4.32 NF-κB luciferase 48 h before infection, and relative light units (RLU) measured 7 h postinfection. For cytokine mRNA measurement, cDNA was prepared at 4 h postinfection. Gene expression assays are listed in *SI Materials and Methods*.

Protein Purification and In Vitro Ubiquitination Reactions. TRIM21, E1, and E2s were expressed in *Escherichia coli* C41 cells as GST-TEV or His-MBP fusion proteins and purified to homogeneity. In vitro ubiquitination reactions were carried out as previously described (4). For His-tag pulldowns, 10^6 TE671 cells, transfected with 6xHis-TRIM21 and HA-Ub, were incubated with Ni^{2+} agarose and purified proteins analyzed by immunoblot (IB). Full details of enzymatic reactions and purification procedures are described in *SI Materials and Methods*.

ACKNOWLEDGMENTS. This work was funded by the Medical Research Council (U105181010) and the European Research Council (281627IAI).

1. McNab FW, Rajsbaum R, Stoye JP, O'Garra A (2011) Tripartite-motif proteins and innate immune regulation. *Curr Opin Immunol* 23(1):46–56.
2. Hauler F, Mallery DL, McEwan WA, Bidgood SR, James LC (2012) AAA ATPase p97/VCP is essential for TRIM21-mediated virus neutralization. *Proc Natl Acad Sci USA* 109(48): 19733–19738.
3. Mallery DL, et al. (2010) Antibodies mediate intracellular immunity through tripartite motif-containing 21 (TRIM21). *Proc Natl Acad Sci USA* 107(46):19985–19990.
4. McEwan WA, et al. (2013) Intracellular antibody-bound pathogens stimulate immune signaling via the Fc receptor TRIM21. *Nat Immunol* 14(4):327–336.
5. Pertel T, et al. (2011) TRIM5 is an innate immune sensor for the retrovirus capsid lattice. *Nature* 472(7343):361–365.
6. El Asmi F, et al. (2014) Implication of PMLIV in both intrinsic and innate immunity. *PLoS Pathog* 10(2):e1003975.
7. Nakasone MA, Livnat-Levanon N, Glickman MH, Cohen RE, Fushman D (2013) Mixed-linkage ubiquitin chains send mixed messages. *Structure* 21(5):727–740.
8. Xia ZP, et al. (2009) Direct activation of protein kinases by unanchored polyubiquitin chains. *Nature* 461(7260):114–119.
9. David Y, Ziv T, Admon A, Navon A (2010) The E2 ubiquitin-conjugating enzymes direct polyubiquitination to preferred lysines. *J Biol Chem* 285(12):8595–8604.
10. Hofmann RM, Pickart CM (1999) Noncanonical MMS2-encoded ubiquitin-conjugating enzyme functions in assembly of novel polyubiquitin chains for DNA repair. *Cell* 96(5): 645–653.
11. Wickliffe KE, Lorenz S, Wemmer DE, Kuriyan J, Rape M (2011) The mechanism of linkage-specific ubiquitin chain elongation by a single-subunit E2. *Cell* 144(5):769–781.
12. Christensen DE, Brzovic PS, Klevit RE (2007) E2-BRCA1 RING interactions dictate synthesis of mono- or specific polyubiquitin chain linkages. *Nat Struct Mol Biol* 14(10): 941–948.
13. Shin DY, Lee H, Park ES, Yoo YJ (2011) Assembly of different length of polyubiquitins on the catalytic cysteine of E2 enzymes without E3 ligase; a novel application of non-reduced/reduced 2-dimensional electrophoresis. *FEBS Lett* 585(24):3959–3963.
14. Rajsbaum R, et al. (2014) Unanchored K48-linked polyubiquitin synthesized by the E3-ubiquitin ligase TRIM6 stimulates the interferon-IKKε kinase-mediated antiviral response. *Immunity* 40(6):880–895.
15. Lee MJ, Lee BH, Hanna J, King RW, Finley D (2011) Trimming of ubiquitin chains by proteasome-associated deubiquitinating enzymes. *Mol Cell Proteomics* 10(5):R110.003871.
16. Jacobson AD, et al. (2009) The lysine 48 and lysine 63 ubiquitin conjugates are processed differently by the 26 s proteasome. *J Biol Chem* 284(51):35485–35494.
17. Nathan JA, Kim HT, Ting L, Gygi SP, Goldberg AL (2013) Why do cellular proteins linked to K63-polyubiquitin chains not associate with proteasomes? *EMBO J* 32(4): 552–565.
18. Cooper EM, et al. (2009) K63-specific deubiquitination by two JAMM/MPN+ complexes: BRISC-associated Brcc36 and proteasomal Poh1. *EMBO J* 28(6):621–631.
19. Mansour W, et al. (2015) Disassembly of Lys11 and mixed linkage polyubiquitin conjugates provides insights into function of proteasomal deubiquitinases Rpn11 and Ubp6. *J Biol Chem* 290(8):4688–4704.
20. Chau V, et al. (1989) A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein. *Science* 243(4898):1576–1583.
21. Finley D, et al. (1994) Inhibition of proteolysis and cell cycle progression in a multiubiquitination-deficient yeast mutant. *Mol Cell Biol* 14(8):5501–5509.
22. Tatham MH, Plechanovová A, Jaffray EG, Salmen H, Hay RT (2013) Ube2W conjugates ubiquitin to α-amino groups of protein N-termini. *Biochem J* 453(1):137–145.
23. Scaglione KM, et al. (2013) The ubiquitin-conjugating enzyme (E2) Ube2w ubiquitinates the N terminus of substrates. *J Biol Chem* 288(26):18784–18788.
24. Vittal V, et al. (2015) Intrinsic disorder drives N-terminal ubiquitination by Ube2w. *Nat Chem Biol* 11(1):83–89.
25. Worden EJ, Padovani C, Martin A (2014) Structure of the Rpn11-Rpn8 dimer reveals mechanisms of substrate deubiquitination during proteasomal degradation. *Nat Struct Mol Biol* 21(3):220–227.
26. Yao T, Cohen RE (2002) A cryptic protease couples deubiquitination and degradation by the proteasome. *Nature* 419(6905):403–407.
27. Dantuma NP, Lindsten K, Glas R, Jellne M, Masucci MG (2000) Short-lived green fluorescent proteins for quantifying ubiquitin/proteasome-dependent proteolysis in living cells. *Nat Biotechnol* 18(5):538–543.
28. Palombella VJ, Rando OJ, Goldberg AL, Maniatis T (1994) The ubiquitin-proteasome pathway is required for processing the NF-kappa B1 precursor protein and the activation of NF-kappa B. *Cell* 78(5):773–785.
29. Verma R, et al. (2002) Role of Rpn11 metalloprotease in deubiquitination and degradation by the 26S proteasome. *Science* 298(5593):611–615.
30. Hao R, et al. (2013) Proteasomes activate aggresome disassembly and clearance by producing unanchored ubiquitin chains. *Mol Cell* 51(6):819–828.
31. Beck F, et al. (2012) Near-atomic resolution structural model of the yeast 26S proteasome. *Proc Natl Acad Sci USA* 109(37):14870–14875.
32. Lander GC, et al. (2012) Complete subunit architecture of the proteasome regulatory particle. *Nature* 482(7384):186–191.
33. Pathare GR, et al. (2014) Crystal structure of the proteasomal deubiquitylation module Rpn8-Rpn11. *Proc Natl Acad Sci USA* 111(8):2984–2989.
34. Matyskiela ME, Lander GC, Martin A (2013) Conformational switching of the 26S proteasome enables substrate degradation. *Nat Struct Mol Biol* 20(7):781–788.
35. Zhang Z, et al. (2013) The E3 ubiquitin ligase TRIM21 negatively regulates the innate immune response to intracellular double-stranded DNA. *Nat Immunol* 14(2):172–178.
36. Gack MU, et al. (2007) TRIM25 RING-finger E3 ubiquitin ligase is essential for RIG-I-mediated antiviral activity. *Nature* 446(7138):916–920.
37. Yoshimi R, et al. (2009) Gene disruption study reveals a nonredundant role for TRIM21/Ro52 in NF-kappaB-dependent cytokine expression in fibroblasts. *J Immunol* 182(12):7527–7538.
38. Treier M, Staszewski LM, Bohmann D (1994) Ubiquitin-dependent c-Jun degradation in vivo is mediated by the delta domain. *Cell* 78(5):787–798.
39. Duncan LM, et al. (2006) Lysine-63-linked ubiquitination is required for endolysosomal degradation of class I molecules. *EMBO J* 25(8):1635–1645.