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Regulation of Endoplasmic Reticulum-associated Degradation by RNF5-dependent Ubiquitination of JNK-associated Membrane Protein (JAMP)*

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Clearance of misfolded proteins by endoplasmic reticulum (ER)-associated degradation (ERAD) requires concerted activity of chaperones, adaptor proteins, ubiquitin ligases, and proteasomes. RNF5 is a ubiquitin ligase anchored to the ER membrane implicated in ERAD via ubiquitination of misfolded proteins. Among RNF5-associated proteins is JNK-associated membrane protein (JAMP), a 7-transmembrane protein located within the ER membrane that facilitates degradation of misfolded proteins through recruitment of proteasomes and ERAD regulatory components. Here we demonstrate that RNF5 associates with JAMP in the ER membrane. This association results in Ubc13-dependent RNF5-mediated noncanonical ubiquitination of JAMP. This ubiquitination does not alter JAMP stability but rather inhibits its association with Rpt5 and p97. Consequently, clearance of misfolded proteins, such as CFTRΔ508 and T cell receptor α, is less efficient, resulting in their greater accumulation. Significantly, the RNF5 effect on JAMP is seen prior to and after ER stress response, thereby highlighting a novel mechanism to limit ERAD and proteasome assembly at the ER, to the actual ER stress response.

Degradation of misfolded proteins is part of a complex quality control system that clears diverse proteins independent of sequence or functional similarity (1–3). The unfolded protein response reduces the burden caused by unfolded protein accumulation in the endoplasmic reticulum (ER)[2] (4), in part by activating its key regulatory proteins IRE1-XBP1 and ATF6, which results in transcriptional activation of genes important for unfolded protein response, including components of the ER-associated degradation system (ERAD) (5).

ERAD is regulated by an ER quality control system that marks proteins that cannot fold or assemble into multiprotein complexes for ubiquitin-dependent degradation (1–3). This system consists of molecular chaperones such as BiP (1–3), which interacts with misfolded protein to enable their transfer across the ER membrane via the multispanning membrane proteins Derlin-1/2/3 and Sec61(6), and the AAA (ATPase p97 (also known as VCP or cdc48)). Subsequent to translocation, misfolded proteins are ubiquitinated via ER-anchored ubiquitin ligases, such as the vertebrate gp78, Parkin, RNF5/RMA1, and Hrd1 (7–10), followed by proteasome-mediated degradation.

RNF5 (RING finger domain E3 ligase; also known as RMA1) is one of the few ER-associated E3 ubiquitin ligases. Anchored to the ER membrane via its C terminus, RNF5 consists of a classic RING domain (which confers ligase activity), a single transmembrane-spanning domain located within the C-terminal region, and a formin-like homology domain. RNF5 has been shown to promote degradation of misfolded proteins, such as mutant CFTR (CFTRΔ508) (9, 10). Intriguingly, RNF5-mediated degradation of mutant CFTR requires cooperation with another ligase, as has also been shown for gp78 or CHIP, suggesting a two-stage process in which the first ligase promotes substrate mono-ubiquitination, whereas the second mediates poly-ubiquitination (9, 10).

Interestingly, independent of a role in recognition and ubiquitination of misfolded proteins, RNF5 has been shown to affect both the stability and localization of cytoskeletal proteins in worms and mammals. Studies from our lab identified RNF5 as a ubiquitin ligase targeting the *Caenorhabditis elegans* LIM domain protein UNC-95 for degradation (11). In the muscle, UNC-95 is important for the assembly of dense bodies and M-lines attachment structures that anchor actin-containing thin filaments and myosin-containing thick filaments, respectively, to the muscle cell membrane. The *C. elegans* dense bodies are similar to focal adhesions both in their structure and function (12). In fact, RNF5 was found to affect the LIM and LD domain protein paxillin in human and mouse cells (13). Unlike Unc95 degradation in worms, RNF5 ubiquitination of paxillin results in its translocation from the focal adhesion complex to the cytosol, with a concomitant effect on cell migration and adhesion (13). Thus, RNF5 regulation of cytoskeletal proteins results in either canonical ubiquitination and degradation or noncanonical ubiquitination (Ubc13-dependent, non-Lys-48 polyubiquitin chains), which alters protein localization and likely alters cell signaling. Consistent with our observations in *C. elegans*, work performed using RNF5 transgenic and knockout mice confirmed a role of RNF5 in skeletal muscle. Based on histology and pathology criteria, inducible RNF5 expression resulted in degenerative myopathy with characteristic pheno-

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[2] The abbreviations used are: ER, endoplasmic reticulum; ERAD, ER-associated degradation; JAMP, JNK-associated membrane protein; JNK, c-Jun N-terminal kinase; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating protein; E3, ubiquitin ligase; CFTR, cystic fibrosis transmembrane regulator; shRNA, short hairpin RNA; CHIP, C terminus of HSC70-interacting protein; WT, wild type; TBS, Tris-buffered saline; DTT, dithiothreitol; HA, hemagglutinin; PBS, phosphate-buffered saline; IBM, inclusion body myocytis; TCRα, T cell receptor α.

types seen in inclusion body myocytis (IBM (14)), including high levels of ER stress. In humans, sporadic IBM is the more common myopathy affecting older patients and is marked by progressive muscle weakness associated with inflammatory response, vacuolar degeneration, and aggregate formation (15, 16). Accumulating evidence associates sporadic IBM with a high level of ER stress, as part of the etiology of this disease (17).

Among RNF5-associated proteins is the 7-transmembrane ER-anchored JNK-associated membrane protein (JAMP). Under stress conditions JAMP associates with JNK and prolongs its activity (18). JAMP also serves as a receptor for proteasomes in the ER, facilitating ERAD. By associating with proteasome components and ERAD regulatory proteins, including p97 and gp78, JAMP increases degradation of misfolded proteins (19). Because RNF5 and gp78 cooperate in recognizing misfolded proteins (9), and because RNF5 is found in a complex with JAMP, we investigated the significance of such association. We demonstrate that RNF5 controls JAMP by causing its noncanonical ubiquitination and interfering with recruitment of proteasome and ERAD components, thereby attenuating the scaffold protein capacity of JAMP. RNF5 regulation of JAMP occurs primarily prior to and following ER stress, thereby offering a new mechanism for control of the ERAD process.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—Human embryonic kidney 293T and HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% calf serum and antibiotics (penicillin, streptomycin 100 units/ml; Invitrogen) in 5% CO_2 at 37 °C. Transfections were performed using the calcium phosphate technique for 293T cells and Lipofectamine Plus (Invitrogen) for HeLa cells.

Antibodies and Immunoblot Analysis—Polyclonal antibodies against the JAMP N-terminal domain (amino acids 1–58) were generated as described (18) and used for immunoblot analysis (1:500). Antibodies for proteasome subunits (Rpt4 (1:1000) and Rpt5 (diluted 1:5000)) were used as described (20). The following antibodies were purchased from the indicated vendors: monoclonal FLAG antibodies (Sigma; 1:10,000); HA polyclonal antibodies (Santa Cruz Biotechnology; 1:1000); monoclonal Myc antibodies (Santa Cruz Biotechnology; 1:5000); p97 polyclonal antibodies (Bethyl; 1:1000); CFTR monoclonal antibodies (Upstate; 1:1000); Ubc13 antibody (Zymed Laboratories Inc., 1:5000). Antibodies detecting Lys-63 chain ubiquitin were kindly obtained from Dr. V. Dixit at Genentech. Antibodies against RNF5 were generated as described (14) and used for immunoblot analysis (1:2000). Immunoblots were visualized using goat anti-mouse or anti-rabbit Alexa-Fluor 680 secondary antibodies (Molecular Probes) followed by detection with the Odyssey Infrared Imaging System (LI-COR Biosciences).

DNA Constructs—Full-length (936 bp) mouse *JAMP* cDNA was amplified by PCR and cloned into BamHI/XhoI sites of pcDNA-FLAG and pEF-hemagglutinin (HA). HA-TCRα and p97 constructs were gifts from Dr. R. Kopito (21). FLAG-Rpt4 and -Rpt5 constructs were gifts from Dr. Keiji Tanaka. FLAG- or Myc-tagged full-length human RNF5 was constructed in pEF-FLAG vector or myc-pCDNA3 vectors, respectively. RNF5 was deleted of its C-terminal domain via PCR-based cloning into pEF-FLAG of the DNA fragment corresponding to amino acids 1–164. A RING domain deletion mutant of human RNF5 (from 49 to 268 bp) was generated via PCR-based cloning. CFTR constructs were kindly provided by Dr. Gergely Lukacs. HA-tagged, FLAG-tagged Ubc13 WT or dominant negative constructs (C87A), HA-tagged WT ubiquitin, and HA-tagged ubiquitin mutants (K48R, K63R, K48R/K63R, KO48, and KO63) were generated as described (22, 23).

Gene Silencing by RNA Interference—Silencing of RNF5 in HeLa cells and generation of stable cell lines were performed using the pSuper and pCMS3-cherry construct (kind gift from Dr. Dan Billadeau (24)) as described previously (14). Levels of RNF5 expression were monitored by Western blot and reverse transcription-PCR analysis using the corresponding primers.

Immunoprecipitation—Unless otherwise specified, coimmunoprecipitations were performed following extraction of proteins with 1% Triton X-100 in 50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin A. Where specified in the figure legends, immunoprecipitations were performed under mild conditions (following protocol for immunopurification of endogenous proteasomes from cells using sonication and lysis buffer (50 mM Tris, pH 7.4, 10% glycerol, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, and 10 μg/ml aprotinin)). In all cases, samples were centrifuged (15 min, 14,000 rpm), and supernatants were incubated for 1 h with the indicated antibodies (2 μg). Immunoprecipitation was performed by incubation (40 min at 4 °C) with protein G-agarose (Invitrogen). After washings with lysis buffer, proteins were solubilized in 3× Laemmli buffer and separated on SDS-PAGE followed by immunoblot analysis with the indicated antibodies.

Immunohistochemistry—HeLa cells grown on coverslips (22-mm^2, Chase Scientific Glass) were fixed using freshly prepared 3% paraformaldehyde in PBS (5 min at room temperature). Cells were then washed (three times, 5 min each) in PBS, followed by permeabilization in 0.1% Triton X-100 in PBS, pH 7.4, for 1 min and an additional three 5-min washes in PBS. Cells were then incubated in PBS supplemented with 3% bovine serum albumin for 30 min. Cells were incubated with antibodies (1 h at room temperature) in a humidity chamber and then washed in PBS (three times, 5 min each) before incubation with 100 μl of Alexa-488- and Alexa-568-conjugated anti-rabbit or anti-mouse immunoglobulin G (Molecular Probes) diluted (2 μg/ml) in PBS containing 0.2% bovine serum albumin (60 min at room temperature in a light-protected humidity chamber). Cells were rinsed three times in PBS. Coverslips were mounted on glass slides using Vectashield (Vector Laboratories). For IHC, antibodies were used at the following concentrations: Rpt6 1:100; Rpt2 (1:100); 20 S (1:100); FLAG (4.6 μg/ml); HA (2 μg/ml); and calnexin (1:100). Detection of JAMP by IHC requires a different fixation method (methanol) than that used for other proteins (Triton X-100), thereby requiring us to perform parallel rather than coimmunostaining. Immunofluorescence data were obtained using Olympus TH4–100 microscope and Slidebook 4.1 digital microscopy software. Images

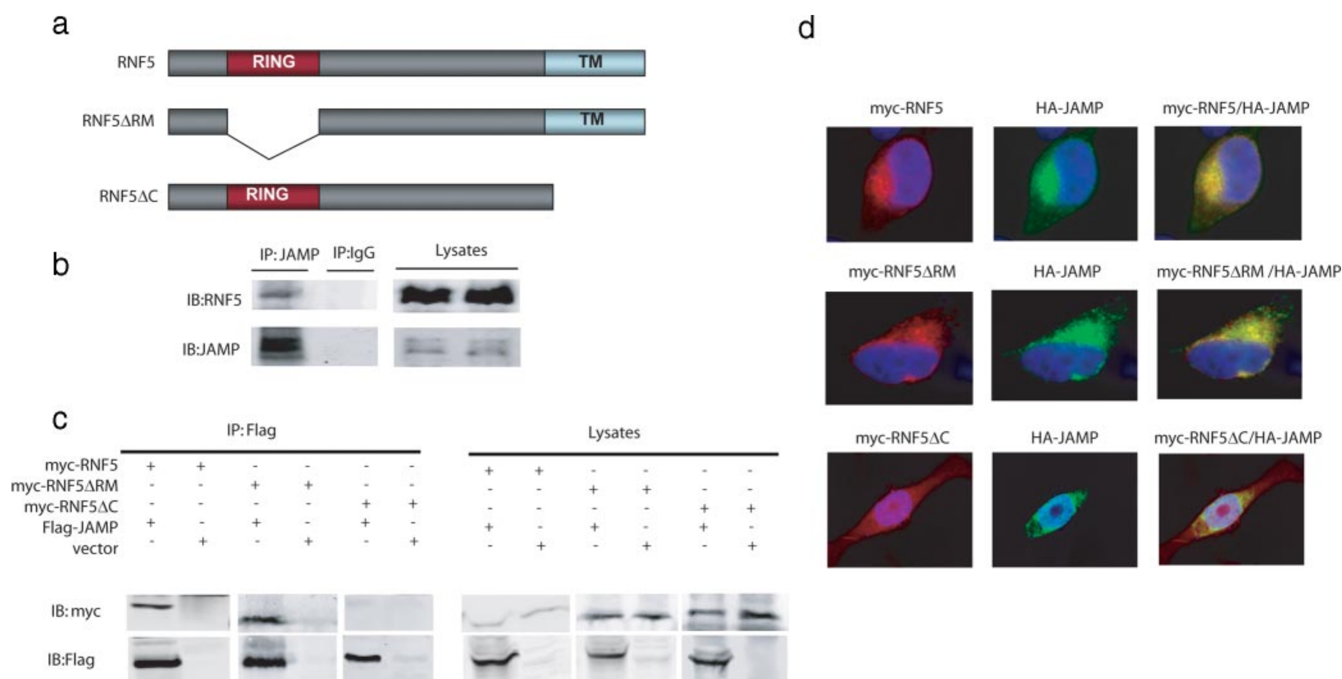

FIGURE 1. **RNF5 association with JAMP requires an intact membrane anchoring domain.** *a,* schematic representation of RNF5 constructs. RING mutant RNF5 was generated by deletion of a region encoding 78 amino acids of the RING finger domain. C-terminally truncated RNF5 was created by deleting a region encoding the last 16 amino acids of the protein that contains the transmembrane (*TM*) region. *b,* JAMP interacts with endogenous RNF5. JAMP was immunoprecipitated (*IP*) from 293T cells following immunoblot (*IB*) analysis against RNF5 (*upper*) or JAMP (*lower*). *c,* interaction of JAMP with RNF5 requires membrane anchoring. Either the full-length protein, the RING finger deletion mutant, or a C-terminally truncated, Myc-tagged RNF5 were cotransfected with FLAG-JAMP into 293T cells. Proteins were prepared as in *b* and immunoprecipitated with FLAG antibodies followed by immunoblot analysis with Myc antibody or FLAG antibodies as noted in the Western blots shown. *d,* JAMP colocalizes with full-length RNF5 but not C-terminally truncated protein. HA-JAMP and FLAG-tagged, full-length RNF5 or C-terminally truncated RNF5 were cotransfected into HeLa cells. Cells were fixed and immunostained with HA and FLAG antibodies after 24 h.

were deconvoluted using constrained iterative and nearest neighbor algorithms.

Cycloheximide Chase Analysis—Cells (293T) were transfected with indicated plasmids and 24 h later subjected to cycloheximide treatment at 20 μg/ml. Proteins were lysed in detergent-based buffer at indicated time points. Analysis was performed following immunoprecipitation of misfolded proteins (for CFTRΔ508 with HA antibodies) or by Western blot (for HA-TCRα).

In Vitro Ubiquitination—Cells were transfected with indicated plasmids, and JAMP was purified by immunoprecipitation as described above with anti-HA antibodies using protein G beads. After extensive washing, bead-bound material was resuspended in 50 mM HEPES, pH 7.6, 5 mM $MgCl_2$, 1 mM ATP, 1 mM DTT prior to addition of infrared labeled ubiquitin and the indicated recombinant ubiquitin system enzymes. After incubating the reactions for 60 min at 37 °C with mixing, bead-bound material was washed three times with 25 mM HEPES, pH 7.6, 300 mM NaCl, 0.2% Triton X-100, 2 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT prior to analysis by reducing SDS-PAGE. Recovered JAMP was assessed by anti-HA immunoblotting and associated ubiquitin by direct imaging on a Licor Odyssey. The final concentrations per reaction were as follows: 1 μM ubiquitin, 100 nM E1, and 500 nM E2. The preparation of infrared labeled ubiquitin, E1, and E2 enzymes from recombinant sources will be described elsewhere.

In Vivo Ubiquitination—Cells were transfected with indicated plasmids and HA-tagged ubiquitin as described previously (22, 23). Harvested cells were lysed in 1 volume of 2% SDS in TBS (10 mM Tris-HCl, pH 8.0) at 95 °C for 10 min. Nine volumes of 1% Triton X-100 and 2 mM EDTA in TBS were added, and lysates were incubated on ice for 30 min, followed by sonication (15 s, three times). The solution was incubated for 30 min at 4 °C with protein G beads (Invitrogen) and clarified by 30 min of centrifugation (14,000 rpm) at 4 °C. The protein concentration was determined by the Bradford assay. For immunoprecipitation, 1 mg of protein was incubated with anti-FLAG or anti-Myc antibodies at 4 °C overnight before protein G beads were added for 2 h. Beads were washed once with TBS, 1% Triton X-100, 1% SDS, twice with 0.5 M LiCl, TBS, 1% Tris-NaCl, and again in PBS, 1% Triton X-100. Proteins were loaded onto 8–15% SDS-polyacrylamide gels and immunoblotted with indicated antibodies.

RESULTS

JAMP Interacts with RNF5 at the ER Membrane—Yeast two-hybrid screening identified JAMP as an RNF5-interacting protein (18). Because both RNF5 and JAMP function in ERAD, we assessed the function of the RNF5/JAMP interaction. We first examined interaction requirements *in vivo*. Full-length RNF5 or truncated forms lacking either the RING domain (required for ubiquitin ligase activity) or the C terminus, which is required for ER membrane localization primarily, were generated (Fig. 1*a*) and assessed for association with JAMP. Endogenous RNF5 associated with endogenous JAMP (Fig. 1*b*), confirming two-hybrid results. We next asked whether truncated forms of RNF5 retained the ability to associate with JAMP. Although deleting the RING finger domain did not impede

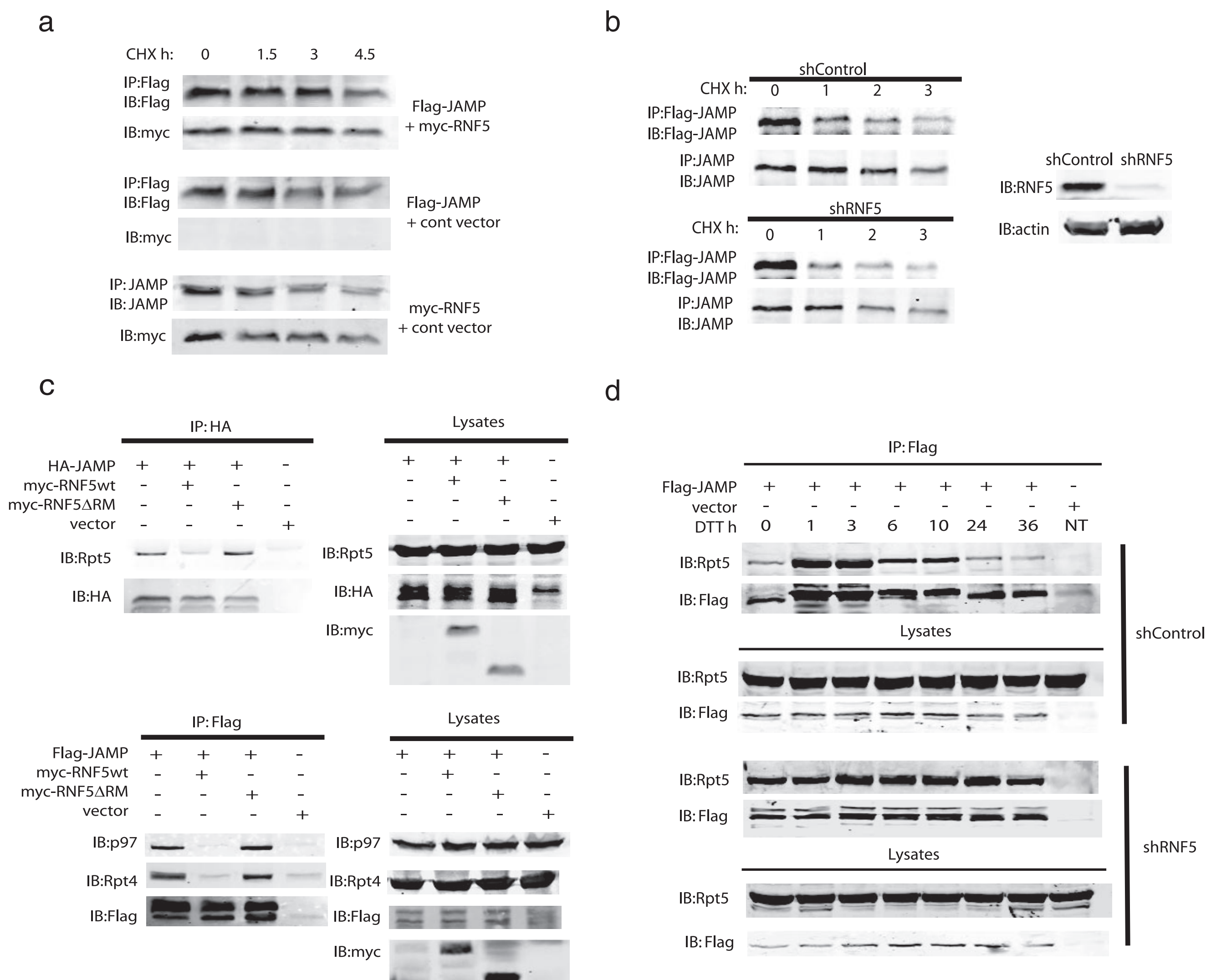

FIGURE 2. **RNF5 ligase activity decreases JAMP association with proteasome components without altering JAMP stability.** *a,* overexpression of RNF5 does not alter half-life of endogenous or overexpressed JAMP. FLAG-JAMP, myc-RNF5, or both were cotransfected into 293T cells, and cells were treated with cycloheximide (*CHX*) (20 μg/ml) for the indicated times. Extracts were immunoprecipitated (*IP*) with FLAG or JAMP antibodies and processed for immunoblot (*IB*) analysis with indicated antibodies. *b,* inhibition of RNF5 expression does not alter the half-life of JAMP. FLAG-JAMP was transfected into HeLa cells stably expressing shRNAs of control (shControl) or RNF5 (*shRNF5*). After 24 h cells were treated with cycloheximide for indicated time points. Protein extracts were immunoprecipitated with FLAG antibodies and processed for immunoblot analysis with indicated antibodies. In parallel, endogenous JAMP was immunoprecipitated using JAMP antibody from control (*shControl*) or RNF5 (*shRNF5*) expressing cells after cycloheximide treatment. Immunoprecipitates were subjected to immunoblot analysis with JAMP antibody. *Lower panel* depicts expression of RNF5 in the cells stably expressing control or RNF5 shRNA. *c,* overexpression of RNF5 disrupts interaction between JAMP and the 19 S proteasome subunits Rpt4 and Rpt5 and the ERAD component p97. 293T cells were cotransfected with FLAG-JAMP and Myc-tagged full-length RNF5, the Myc-tagged RING deletion mutant RNF5, or vector. After 36 h proteins were prepared using lysis buffer supplemented with 1% Triton X-100. Lysates were immunoprecipitated with antibodies against FLAG followed by immunoblot with antibodies for endogenous Rpt4 (*right panel*), Rpt5 (*left panel*), p97 (*right panel*), FLAG, and Myc. *d,* down-regulation of endogenous RNF5 enhances interaction between JAMP and the 19 S proteasome particle. HA-JAMP was transfected into shControl and shRNF5 HeLa cell lines and treated with 10 mM DTT for 30 min. At indicated times, cells were lysed as indicated in *b* and immunoprecipitated with HA antibodies followed by immunoblot analysis with Rpt5 and HA antibodies.

RNF5-JAMP binding, deletion of the C terminus abrogated the association (Fig. 1*c*), indicating that membrane anchoring of RNF5 is required for JAMP association. Because both proteins localize primarily at the ER, these data suggest that RNF5 association with JAMP occurs at the ER membrane. Consistent with these findings, colocalization of RNF5 and JAMP was seen with full-length but not with C-terminally deleted RNF5 (Fig. 1*d*).

RNF5 Regulates JAMP Association with Proteasome Subunits— We next assessed the functional significance of the RNF5/JAMP interaction. Because the RNF5 ligase promotes degradation of some substrates, we investigated whether RNF5 alters the JAMP half-life. Cycloheximide chase experiments revealed that RNF5 misexpression did not decrease the half-life of exogenous or endogenous JAMP (Fig. 2*a*). Similarly, inhibition of RNF5 expression by a specific shRNA did not alter half-life of endogenous or ectopically expressed JAMP (Fig. 2*b*). Because RNF5 also alters protein function by noncanonical ubiquitination (*i.e.* of paxillin (13)), we asked whether RNF5 altered JAMP function in ERAD, given that JAMP functions as a receptor for proteasome subunits at the ER. To do so we monitored possible changes in association of JAMP with the proteasome subunit

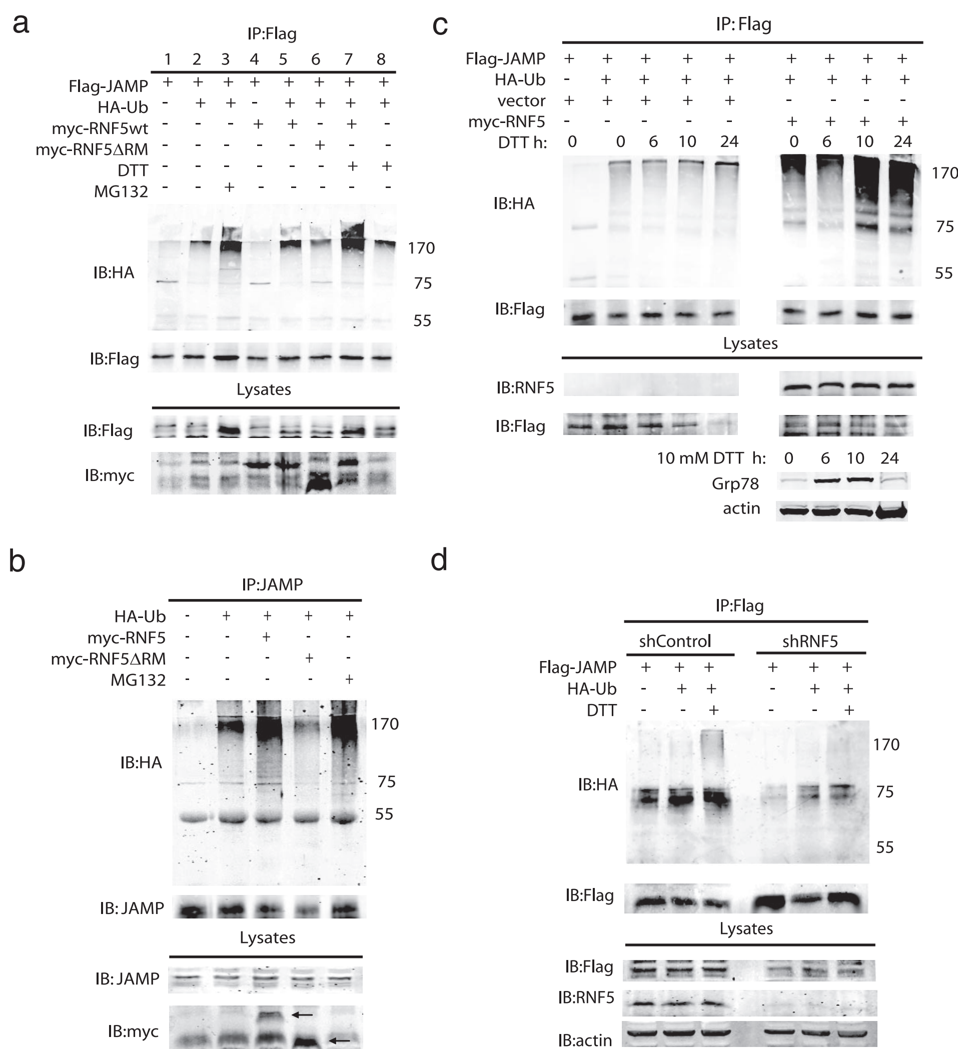

FIGURE 3. **RNF5 induces timely ubiquitination of JAMP.** *a,* overexpressed JAMP is ubiquitinated by RNF5 *in vivo*. FLAG-JAMP was cotransfected with HA-ubiquitin (*HA-Ub*) and full-length RNF5 or the RING domain mutant into 293T cells. After 24 h, cells were treated with 10 mM DTT for 30 min and lysed 24 h later following 5 h of mock or MG132 (40 μM) treatment. Proteins were extracted with 2% SDS/TBS, diluted with 9 volumes of 1% Triton X-100/TBS, and immunoprecipitated (*IP*) with anti-FLAG antibody, followed by immunoblotting (*IB*) with anti-HA antibodies. The blot was reprobed with anti-FLAG antibodies. *b,* endogenous JAMP is ubiquitinated by RNF5 *in vivo*. Myc-tagged full-length or RING mutant forms of RNF5 were cotransfected with HA-ubiquitin into 293T cells. After 24 h cells were treated with MG132 for 5 h as indicated. Proteins were extracted with 2% SDS/TBS, diluted with 9 volumes 1% Triton X-100/TBS, and immunoprecipitated with anti-JAMP antibody, followed by immunoblot analysis with anti-HA antibodies. The blot was reprobed with anti-JAMP antibodies. *c,* effect of RNF5 on JAMP ubiquitination is increased following relief from the ER stress. FLAG-JAMP was cotransfected into 293T cells with HA-ubiquitin and vector only or full-length RNF5. Cells were treated with DTT (10 mM for 30 min) and lysed at time points indicated in *a*. Protein extracts were subjected to immunoprecipitation with anti-FLAG antibody, followed by immunoblot analysis with anti-HA. The blot was reprobed with anti-FLAG antibodies. Lysates were also probed for Grp78 expression as a control for ER stress response. *d,* RNF5 is responsible for JAMP ubiquitination after ER stress. HeLa cells stably expressing shControl or shRNF5 were cotransfected with FLAG-JAMP with and without HA-ubiquitin and treat as indicated with DTT (10 mM for 30 min). After 24 h proteins were extracted as detailed in *a*, and immunoblots were performed with indicated antibodies.

Rpt5 in the presence or absence of RNF5. Notably, overexpression of WT but not a RING mutant form of RNF5 inhibited JAMP association with Rpt5, Rpt4, and p97 (Fig. 2*c*). Similarly, association between JAMP and other proteasome subunits Rpt6 was decreased upon RNF5 expression (data not shown). These data reveal that RNF5 regulates JAMP association with proteasome subunits, an activity that requires ubiquitin ligase activity. To confirm the effect of RNF5 on JAMP association with proteasome subunits, we inhibited RNF5 expression by corresponding shRNA and assessed possible changes in interaction with the proteasome. Cells stably expressing RNF5 shRNA and exhibiting >70% inhibition of RNF5 expression were established and characterized (14). Rpt5/JAMP association was higher in cells with knocked down RNF5 expression (Fig. 2*d*, *time 0*) and was not altered in response to ER stress, compared with the shRNA control cells (Fig. 2*d*, *1–36-h* time points). Association between Rpt5 and JAMP was largely unchanged during the response to ER stress (3–6 h) in shRNF5-expressing cells (Fig. 2*d*, *shRNF5 panels*). These findings suggest that RNF5 control of JAMP-Rpt5 association primarily takes place prior to and after but not during ER stress.

RNF5 Causes Noncanonical JAMP Ubiquitination Using Lys-63 Chain Topology—The effect of RNF5 on JAMP association with Rpt5 rather than JAMP stability implies that RNF5 ubiquitination of JAMP may be noncanonical. We thus assessed JAMP ubiquitination by RNF5 *in vivo*. Ectopically expressed JAMP was efficiently ubiquitinated *in vivo* by WT but not the RING mutant form of RNF5 (Fig. 3*a*, compare *lane 2* with *lanes 5* and *6*). Consistent with these findings, analysis of endogenous JAMP ubiquitination revealed JAMP ubiquitination upon expression of WT but not the RING mutant form of RNF5 (Fig. 3*b*). Importantly, the degree of JAMP ubiquitination was further increased following ER stress in RNF5-expressing cells (Fig. 3*a*, compare *lanes 5* and *7*). Of note, this analysis was carried out 24 h after a short (30 min) ER stress treatment and likely reflects the last phase of or exit from the ER stress response.

Earlier experiments (Fig. 2) pointed to the possibility that JAMP ubiquitination via RNF5 occurs at distinct phases of the ER stress response. We thus monitored possible changes in RNF5-dependent JAMP ubiquitination at different time points after ER stress. Notably, RNF5 elicited ubiquitination of JAMP prior to ER stress (time 0), which was reduced in the first few hours of ER stress (6-h time point), and then increased following ER stress, as seen at 10 and 24 h (Fig. 3*c*). Grp78 expression

serves as a marker for ER stress (Fig. 3*c, lower panel*), confirming that the degree of JAMP ubiquitination by RNF5 primarily occurs prior to and following ER stress response.

We also analyzed possible change in JAMP ubiquitination in cells in which RNF5 expression was inhibited by shRNA. Control cells showed JAMP ubiquitination 24 h after ER stress, but ubiquitination was markedly attenuated in shRNF5-expressing cells (Fig. 3*d*). These findings support the notion that RNF5 mediates JAMP ubiquitination prior to and following ER stress.

Because RNF5 inhibits JAMP association with proteasome subunits rather than JAMP stability, we assessed the topology of ubiquitin chains formed on JAMP. Analysis of ubiquitin mutants that cannot form Lys-48 or Lys-63 chains allows identification whether either topology predominates in the formation of polyubiquitin chains on the substrate (22, 23, 25). Cells were cotransfected with either K48R, K63R, or double K48/63R mutants, and the degree of RNF5-mediated JAMP ubiquitination was assessed. JAMP ubiquitination increased upon expression of the K48R mutant, whereas K63R expression attenuated that ubiquitination (Fig. 4*a*). In agreement, we observed ubiquitination of endogenous JAMP following expression of WT or KO63 mutant ubiquitin (which allows ubiquitin chain formation to take place only through lysine 63), whereas expression of the ubiquitin KO48 mutant (which allow ubiquitin chain formation to take place only through lysine 48) abolished JAMP ubiquitination (Fig. 4*b*). Furthermore, using antibodies that selectively recognize Lys-63 ubiquitin chain topology enabled the selective detection of JAMP but not CFTR ubiquitination (Fig. 4*c*). These findings establish that RNF5 mediates Lys-63-based polyubiquitination of JAMP, consistent with the effect of RNF5 on JAMP complex formation with proteasome subunits.

Because RNF5 catalyzes degradation of misfolded mutant protein CFTR (CFTRΔ508 (9, 10)), we also assessed the topology of CFTR ubiquitination by RNF5. CFTR ubiquitination occurred at the canonical Lys-48 topology, as misexpression of the K48R mutant efficiently inhibited CFTR ubiquitination by RNF5, whereas the K63R mutant did not (Fig. 4*d*). These data suggest that RNF5 elicits canonical ubiquitination and subsequent degradation of the misfolded protein (9, 10) as opposed to noncanonical ubiquitination of JAMP, which affects association with proteasome subunits. The ability of RNF5 to elicit diverse topology of multichain ubiquitin on different substrates raises the possibility that different E2 may be utilized by RNF5 in each of these reactions.

Ubc7 and Ubc13 Are Activated by Coexpression of JAMP and RNF5 in Vitro and Enhance JAMP Ubiquitination in Vivo—We next sought to determine whether the RNF5/JAMP interaction could stimulate the activity of E2s known to be associated with ERAD *in vitro*. After purifying cotransfected JAMP and RNF5 from cells via epitope-tagged JAMP, bead-bound material was resuspended in reaction buffer containing ATP and ubiquitin with E1 and Ubc6, Ubc7, or Ubc13 (Fig. 4*e*). The resulting reactions were incubated for 60 min at 37 °C, and noncovalently bound reaction products were reduced by washing the beads extensively prior to SDS-PAGE. Whereas a slight increase in Ubc6 activity in the presence of RNF5/JAMP was observed (Fig. 4*e*, compare *lanes 2* and *6*), we observed increased ubiquitin incorporation with both Ubc7 and Ubc13 in the presence of RNF5/JAMP relative to negative control reactions (Fig. 4*e*, compare *lanes 3* and *4* with *lanes 7* and *8*). In agreement, coexpression of Ubc7 or Ubc13 enhanced *in vivo* ubiquitination of JAMP (Fig. 4*f*). These data suggest that both Ubc7 and Ubc13 can be activated *in vitro* by RNF5 and JAMP and are able to increase degree of JAMP ubiquitination *in vivo*.

Ubc13 Is Required for RNF5-mediated JAMP Ubiquitination and Association with Proteasome Subunits—We previously demonstrated that the ability of RNF5 to assemble Lys-63 ubiquitin chains depends on functional Ubc13 (13). Here we assessed the effect of Ubc13 on RNF5-mediated JAMP ubiquitination. As shown in Fig. 5*a*, in the presence of RNF5, ER stress caused an increase in JAMP ubiquitination 24 h after treatment. Intriguingly, the level of JAMP ubiquitination markedly increased in cells expressing WT but not mutant Ubc13. As our data also suggest that the RNF5 effect on JAMP is primarily seen prior to and following ER stress, we monitored the effect of Ubc13 on JAMP ubiquitination 24 h after ER stress. The presence of WT Ubc13 promoted more robust JAMP ubiquitination after DTT treatment, whereas the dominant negative Ubc13 mutant abolished this effect (Fig. 5*a*). Consistent with these observations, the ubiquitination of endogenous JAMP attenuated in cells that were inhibited for Ubc13 expression (Fig. 5*b*). These data support the role of Ubc13 in JAMP ubiquitination, particularly at late stages or following ER stress.

Because expression of Ubc13 is required for ubiquitination of JAMP following ER stress, and because RNF5 expression inhibits JAMP association with proteasome subunits, we asked whether Ubc13 expression altered JAMP association with proteasome components. Expression of WT but not a mutant form of Ubc13 in cells expressing endogenous RNF5 was sufficient to inhibit Rpt5 association with JAMP (Fig. 5*c, upper panel*). Of interest, Ubc13 expression also attenuated association of JAMP with p97, an important component of the ERAD response previously shown to associate with JAMP (19). Intriguingly, 3 h following ER stress, when interaction between JAMP, Rpt5, and p97 is reportedly enhanced (19), Ubc13 expression was able to inhibit JAMP association with p97 and Rpt5 (Fig. 5*c*, *middle panel*), implying that Ubc13 availability may be a limiting factor in JAMP ubiquitination following ER stress. Consistent with this possibility, the level of Ubc13 was reduced after ER stress (Fig. 5*b, lower panel*). At 24 h after DTT treatment, which represents a post-ER stress time point, Ubc13 expression still strongly inhibited JAMP association with Rpt5 and p97 (Fig. 5*c*, *lower panel*). These findings suggest that Ubc13 regulates JAMP association with proteasome and ERAD components. Although most of the effect elicited by Ubc13 was seen prior to and after ER stress, *i.e.* at times 0 and 24 h after DTT treatment, Ubc13 retained some capacity to inhibit JAMP association with p97 and Rpt5 during ER stress (*i.e.* at a 3-h time point), further supporting the regulation of JAMP complex assembly by noncanonical ubiquitination. To determine whether the Ubc13 effects on JAMP association with Rpt5 or p97 are RNF5-dependent, we have repeated the analysis in cells expressing control or shRNF5. As shown in Fig. 5*d*, dissociation of p97 and Rpt5 from JAMP was no longer seen in cells expressing shRNF5. These data suggest that RNF5-dependent noncanoni-

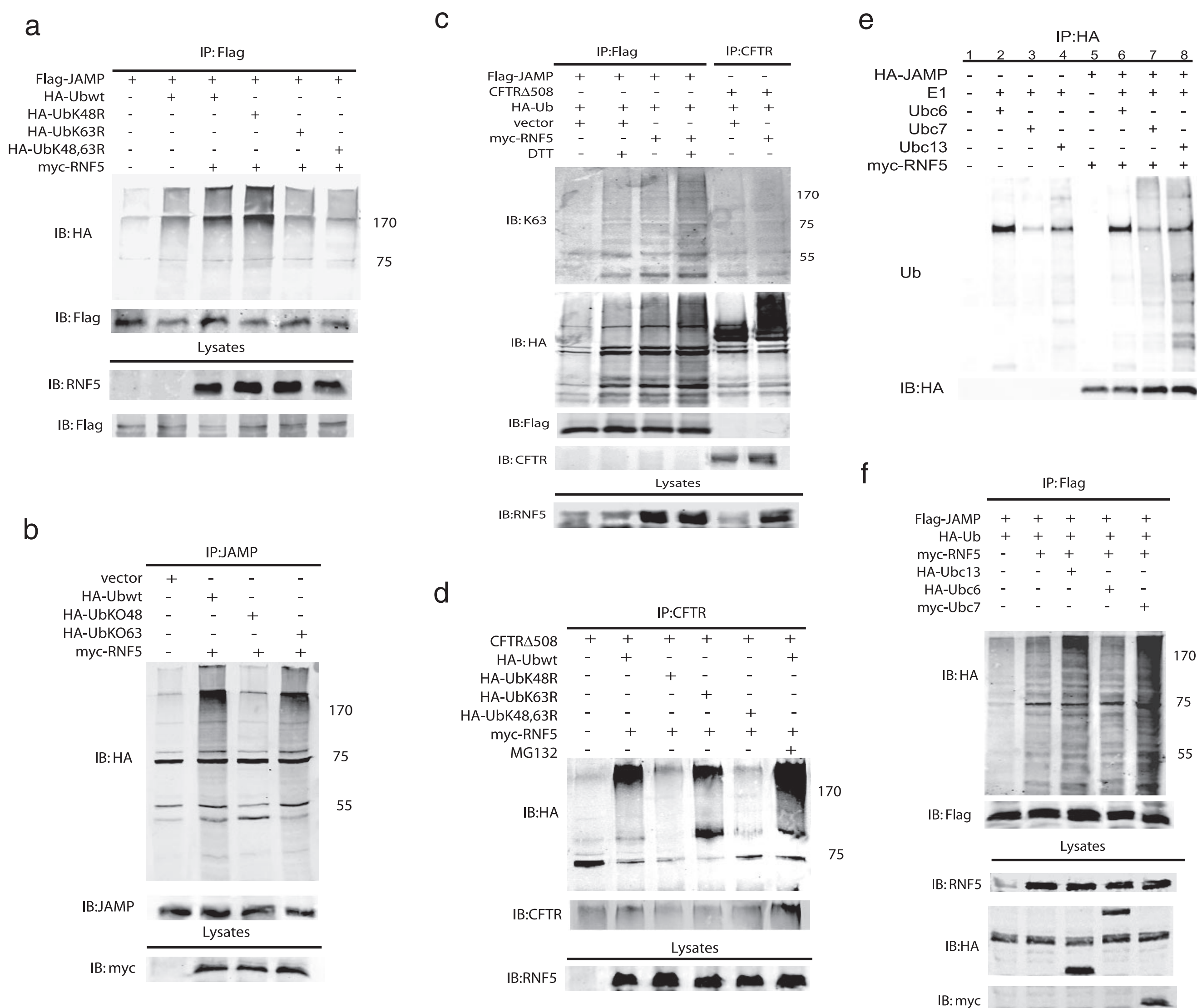

FIGURE 4. **RNF5 induces ubiquitin chains of the Lys-63 topology on JAMP.** *a,* RNF5 mediates ubiquitination of JAMP via Lys-63 chain topology. 293T cells were transfected with FLAG-JAMP with myc-RNF5 and HA-tagged WT or mutant forms of ubiquitin K48R, K63R, K48R/K63R as indicated. After 24 h, proteins were extracted with 2% SDS/TBS, diluted with 9 volumes of 1% Triton X-100/TBS and immunoprecipitated (*IP*) with anti-FLAG antibody, followed by immunoblotting (*IB*) with anti-HA antibodies. The membrane was reprobed with anti-FLAG antibodies. *b,* RNF5 mediates Lys-63-based ubiquitination of JAMP. 293T cells were transfected with FLAG-JAMP, myc-RNF5, and HA-tagged WT or mutant forms of ubiquitin KO48, KO63 as indicated. After 24 h, proteins were extracted with 2% SDS/TBS, diluted with 9 volumes of 1% Triton X-100/TBS, and immunoprecipitated with anti-FLAG antibody, followed by immunoblotting with anti-HA antibodies. *c,* RNF5 mediates Lys-63 chain topology ubiquitination of JAMP and Lys-48 chain topology ubiquitination of CFTRΔ508. 293T cells were transfected with FLAG-JAMP or CFTRΔ508 with myc-RNF5 and HA-tagged WT. Cells were treated with 10 mM DTT for 30 min. After 24 h, proteins were extracted with 6 M urea buffer and processed as indicated (31). JAMP or CFTRΔ508 was immunoprecipitated with anti-FLAG or anti CFTR antibody, respectively, followed by immunoblotting with anti-Lys-63 antibody. The membrane was reprobed with anti-HA, anti-FLAG, and anti-CFTR antibodies. *d,* RNF5 mediates ubiquitination of CFTRΔ508 via Lys-48 chain topology. 293T cells were transfected with CFTRΔ508 with myc-RNF5 and HA-tagged WT or mutant forms of ubiquitin K48R, K63R, K48R/K63R as indicated in *a*. After 24 h, proteins were extracted and analyzed as detailed in *a*. *e,* Ubc7 and Ubc13 function with RNF5/JAMP *in vitro*. 293T cells were either mock-transfected or cotransfected with HA-JAMP and myc-RNF5. Lysates were prepared after 48 h and subjected to anti-HA immunoprecipitation. Bead-bound material was resuspended in reaction buffer containing the indicated proteins, incubated for 60 min at 37 °C, and washed extensively prior to analysis for ubiquitin (*Ub*) incorporation (*top panel*) and JAMP (*bottom panel*). *f,* Ubc7 and Ubc13 enhance JAMP ubiquitination by RNF5 *in vivo*. FLAG-JAMP was cotransfected with HA-ubiquitin, HA-Ubc13, HA-Ubc6, or myc-Ubc7 with or without full-length RNF5 into 293T cells. After 24 h proteins were extracted as in Fig. 3*a* and immunoprecipitated with anti-FLAG antibody, followed by immunoblotting with anti-HA antibodies. The blot was reprobed with anti-FLAG antibodies.

cal ubiquitination of JAMP controls its association with ERAD and proteasome components.

RNF5 Ubiquitination of JAMP Limits Its Role in ERAD—In the ERAD response, JAMP enhances misfolded protein degradation by serving as a platform to recruit ERAD and proteasome components. Hence, changes in JAMP association with these factors could directly alter stability of misfolded proteins. To test this possibility, we monitored changes in JAMP-dependent degradation of the ERAD substrates TCRα and CFTRΔ508 in control *versus* shRNF5-expressing cells. JAMP overexpression effectively reduced steady state levels of TCRα protein (Fig. 6*a*), in agreement with our finding that JAMP facilitates misfolded protein degradation (19). However, cells in which RNF5 expression was inhibited exhibited even more significant decreases in steady state levels of TCRα protein (Fig. 6*a*, compare *3rd* and *4th lanes*), consistent with the observation

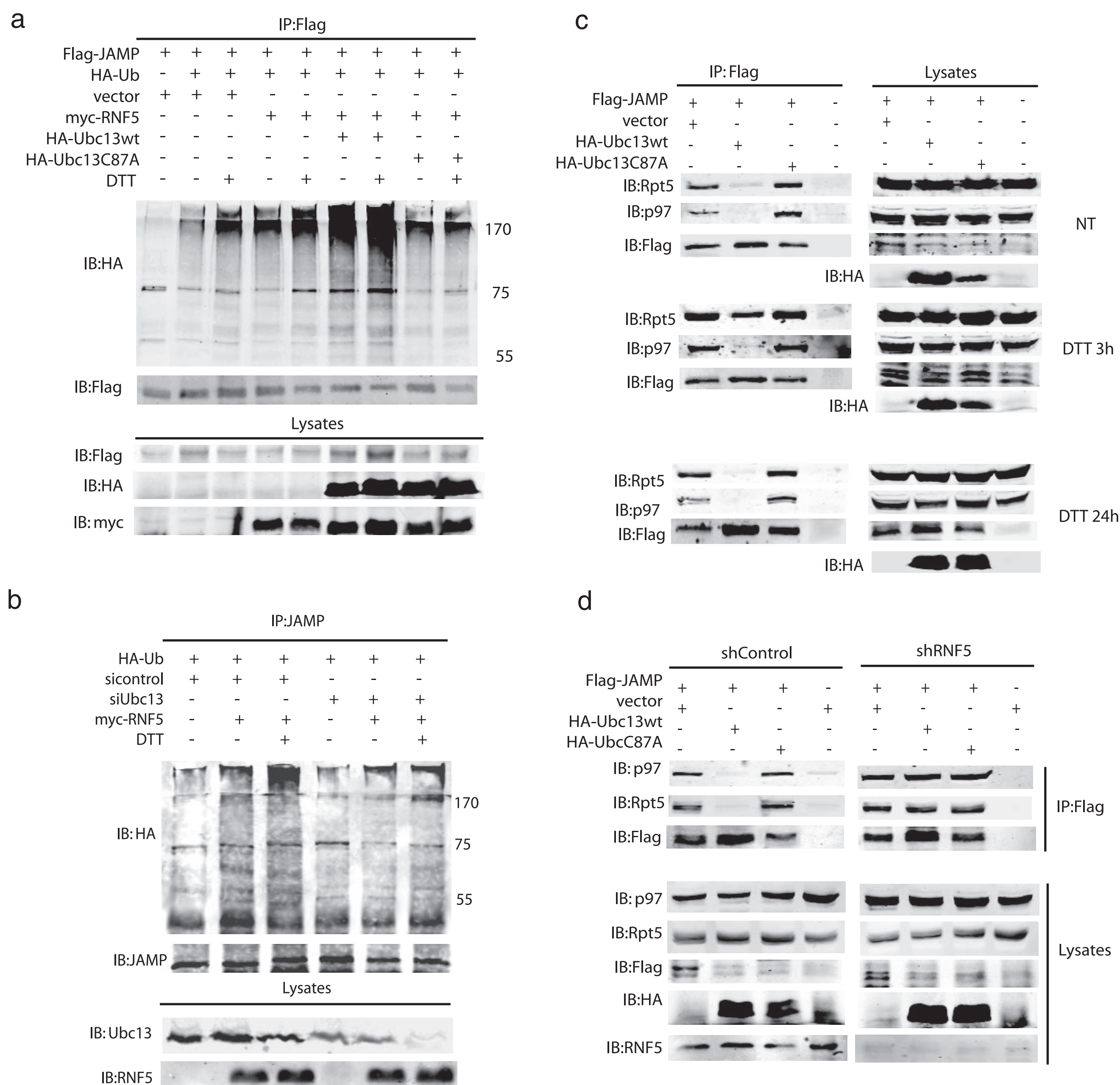

FIGURE 5. **Ubc13 is required for JAMP ubiquitination and decreases JAMP association with Rpt5 and p97.** *a,* Ubc13 increases ubiquitination of JAMP by RNF5 under ER stress conditions. 293T cells were cotransfected with myc-RNF5 and HA-ubiquitin (*HA-Ub*) in the presence of empty vector, HA-Ubc13 WT, or HA-Ubc13C87A mutant. After 24 h cells were treated with DTT (10 mM) for 30 min, and after 24 h proteins were prepared as detailed in Fig. 3*a*. Lysates were immunoprecipitated (*IP*) with FLAG antibodies and immunoblotted (*IB*) with indicated antibodies. *b,* down-regulation of Ubc13 decreases ubiquitination of endogenous JAMP by RNF5. 293T cells were cotransfected with HA-ubiquitin, myc-RNF5, and shControl or shUbc13 vector. After 24 h cells were treated with DTT (10 mM) for 30 min, and 24 h later proteins were prepared as detailed in Fig. 3*a*. Lysates were immunoprecipitated with JAMP antibodies and immunoblotted with indicated antibodies. *c,* overexpression of Ubc13 reduces interaction between JAMP and components of ERAD. 293T cells were cotransfected with FLAG-JAMP, HA-Ubc13 WT, or HA-Ubc13C87A. Cell were treated with DTT (10 mM for 30 min) and lysed at indicated time points. Proteins were extracted using detergent-based buffer (0.1% Triton X-100). Cell lysates were immunoprecipitated with FLAG antibodies. Immunoblot analysis was performed using antibodies to endogenous Rpt5 and p97. *d*, Ubc13 effect on JAMP-Rpt5/p97 association is RNF5-dependent. Experiment was performed as indicated in *c* (*NT panel*) except use of cells that express control or shRNF5.

that association between JAMP and the proteasome subunit Rpt5 increases in cells lacking RNF5 (Fig. 2*d*).

Similarly, CFTRΔ508 half-life was shorter in cells expressing JAMP (Fig. 6*b*, compare *left* and *right panels*) and was further reduced when RNF5 expression was inhibited by shRNA (Fig. 6*b*, compare *upper* and *lower right panels*). These findings suggest that RNF5 ubiquitination of JAMP limit the role of JAMP in ERAD. These data also imply that RNF5 contribution to the degradation of misfolded proteins such as CFTR may depend on availability of JAMP to augment the ERAD process. Because CFTRΔ508 ubiquitination can be mediated by more than one ligase, as proposed for gp78 and CHIP, in addition to RNF5, we tested whether RNF5 may be dispensable for the ubiquitination of CFTRΔ508. Consistent with this idea, inhibition of RNF5 expression did not affect CHIP-dependent ubiquitination of CFTRΔ508, although the steady state level of the CFTRΔ508

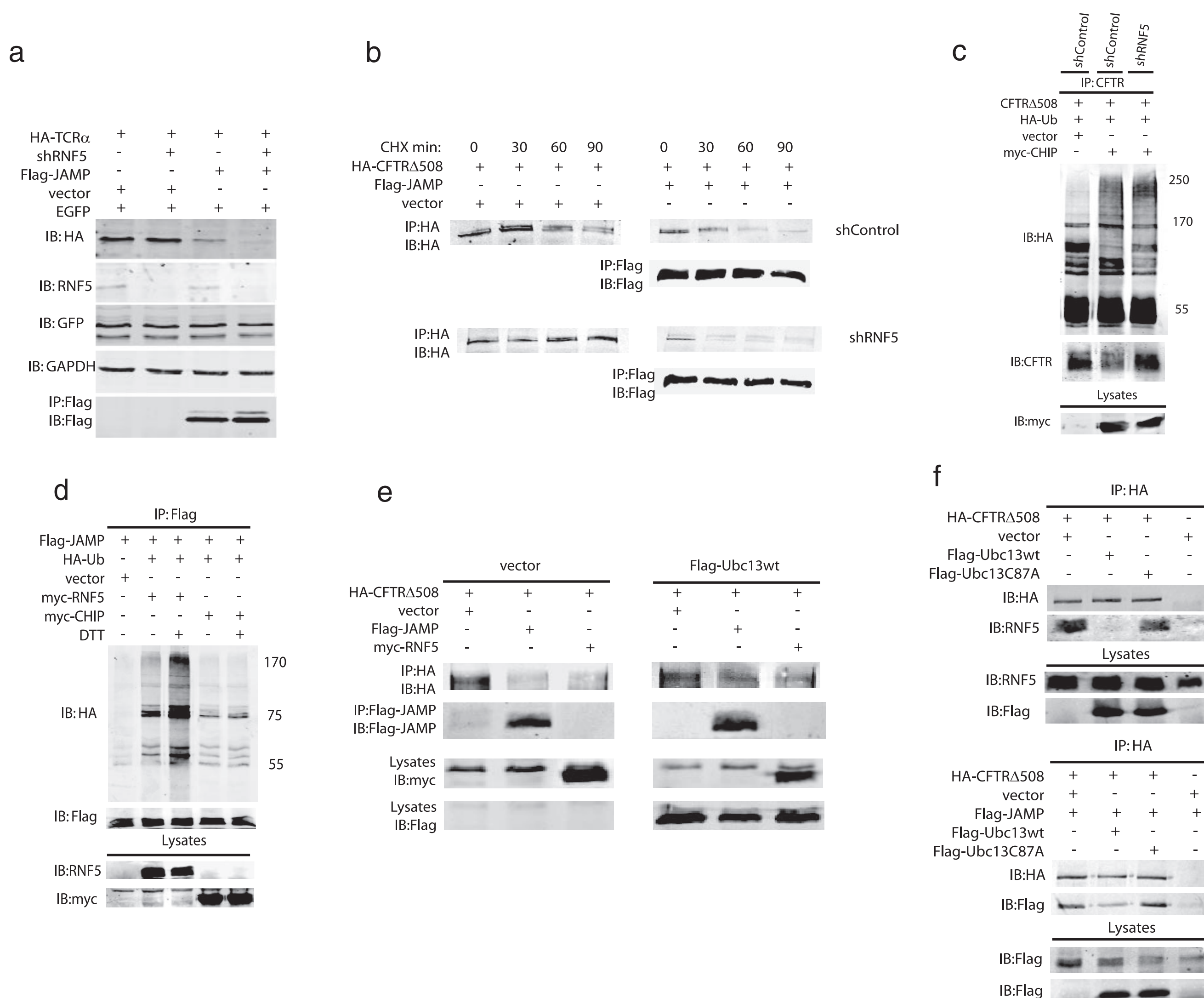

FIGURE 6. **Ubiquitination of JAMP by RNF5 reduces its activity toward ERAD substrates.** *a,* down-regulation of RNF5 facilitates degradation of TCRα by JAMP. TCRα was cotransfected with FLAG-JAMP or vector control into shControl or shRNF5 HeLa cells. Green fluorescent protein (*GFP*) expression served as transfection control. After 36 h cells were lysed, and cell extracts were immunoblotted (*IB*) with indicated antibodies. *EGFP*, enhanced green fluorescent protein; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *IP*, immunoprecipitated. *b,* down-regulation of RNF5 facilitates degradation of CFTRΔ508 by JAMP. CFTRΔ508 was cotransfected with FLAG-JAMP or vector control into shControl or shRNF5 HeLa cells. After 36 h, cycloheximide (*CHX*) (20 μg/ml) was added to the medium. Cells were lysed with detergent-based buffer at indicated time points, and extracts were immunoprecipitated with HA antibodies. Immunoblots were incubated with anti-HA antibodies. Overexpressed JAMP was immunoprecipitated from cells with FLAG antibody and immunoblotted with FLAG antibody. *c,* down-regulation of RNF5 does not alter ubiquitination of CFTRΔ508 by CHIP. CFTRΔ508 was cotransfected with HA-ubiquitin and myc-CHIP into HeLa cells that stably express shControl or shRNF5. After 24 h proteins were extracted as in Fig. 3*a* and immunoprecipitated with anti-CFTR antibody, followed by immunoblotting with anti-HA antibodies. The blot was reprobed with anti-CFTR antibodies. *d,* JAMP is not ubiquitinated by CHIP. FLAG-JAMP was cotransfected with HA-ubiquitin, myc-RNF5, or myc-CHIP into 293T cells. After 24 h, cells were treated with DTT (10 mM for 30 min), and after 24 h proteins were extracted as in Fig. 3*a* and immunoprecipitated with anti-FLAG antibody, followed by immunoblotting with anti-HA antibodies. The blot was reprobed with anti-FLAG antibodies. *e,* overexpression of Ubc13WT attenuates RNF5- or JAMP-dependent degradation of misfolded protein. HA-CFTRΔ508 was cotransfected with FLAG-JAMP or empty vector or Myc-tagged RNF5 with and without WT FLAG-Ubc13. After 24 h cells were lysed and immunoprecipitated with indicated antibodies. Overexpressed RNF5 was detected using anti-Myc antibodies. Overexpression of WT Ubc13 was detected with anti-FLAG antibodies. *f,* overexpression of WT Ubc13 disrupts interaction between ERAD substrates and JAMP or RNF5. CFTRΔ508 was cotransfected with FLAG-JAMP, vector, or Myc-tagged RNF5 with or without FLAG-Ubc13 or FLAG-Ubc13C87A. After 24 h cells were lysed and immunoblotted with indicated antibodies.

did not decrease to the same degree in the absence of RNF5 (Fig. 6*c*). In contrast, ubiquitination of JAMP was not affected by CHIP (Fig. 6*d*). These data provide initial evidence to suggest that the role of RNF5 in ERAD may primarily lie in its control of JAMP function in ERAD.

Given that overexpression of WT Ubc13 directs RNF5 ligase activity toward the Lys-63 chain topology and affects JAMP interaction with ERAD components (Fig. 5), we tested the effect of Ubc13 on CFTRΔ508 degradation. Ubc13 overexpression attenuated CFTRΔ508 degradation, which was otherwise enhanced by expression of either JAMP or RNF5 (Fig. 6*e*). This finding further substantiates the importance of RNF5 ubiquitination of JAMP in ER stress response. Because both RNF5 and JAMP form a complex with ERAD substrates (10, 19), we asked whether Ubc13 expression alters interaction between JAMP, RNF5, and CFTRΔ508. Indeed WT but not the inactive mutant form of Ubc13 perturbed association of CFTRΔ508 with JAMP or RNF5 (Fig. 6*f*). These observations are consistent with the finding that Ubc13 increases the half-life of misfolded protein via its effect on RNF5-dependent ubiquitination of JAMP,

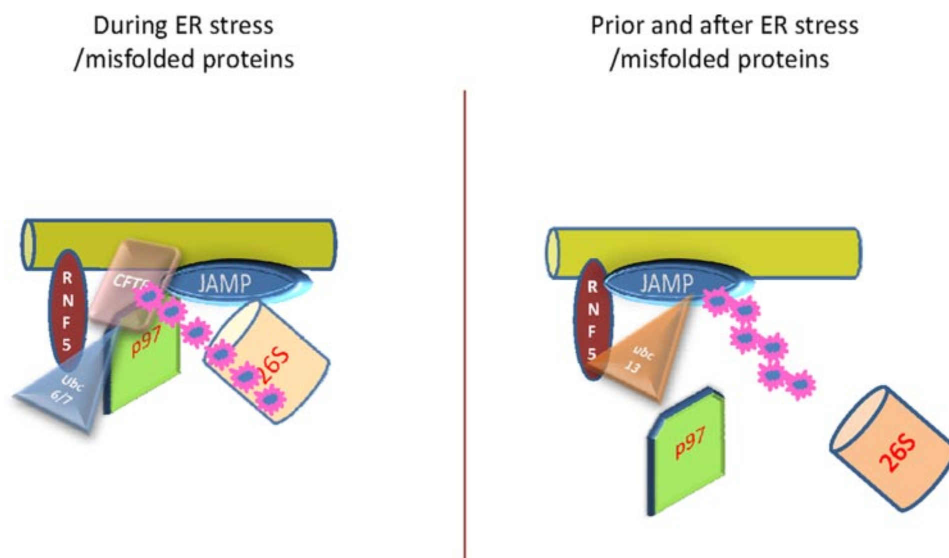

FIGURE 7. **Proposed model for JAMP regulation by RNF5.** Negative regulation of JAMP by RNF5-dependent, noncanonical ubiquitination. RNF5 limits JAMP activity by noncanonical ubiquitination occurring prior to or after the ER stress response, thereby regulating JAMP contribution to ERAD (*right panel*). During ER stress RNF5 mediates canonical ubiquitination of misfolded proteins, as shown for CFTRΔ508, resulting in their degradation and facilitating clearance of misfolded proteins (*left panel*). Timely control of ERAD by positive and negative regulation by RNF5 exemplifies different mechanisms of the ubiquitin system to control ERAD.

which concomitantly decreases association with components of the ERAD response and with proteasomes.

DISCUSSION

Because accumulation of misfolded proteins is toxic and impairs normal cellular homeostasis, as often seen in pathological conditions, the understanding of mechanisms that clear these proteins, such as ERAD, is of great importance and interest.

Among proteins implicated in ERAD is the ER-anchored RING finger E3 ubiquitin ligase RNF5 and JAMP, a 7-transmembrane protein serving as receptor for proteasomes at the ER. This study demonstrates the role of RNF5 in regulating the contribution of JAMP to ERAD and offers novel insight into ERAD control. Our data reveal that RNF5 associates with JAMP within the ER membrane, resulting in Ubc13-dependent, noncanonical ubiquitination of JAMP using the Lys-63 chain topology. Our data demonstrate that such ubiquitination does not alter JAMP stability, but rather it decreases its association with proteasome subunits and p97, a key component of the ERAD response. Consequently, the contribution of JAMP to ERAD is impaired, and misfolded proteins accumulate. These findings reveal a novel mechanism of ERAD by regulating the contribution of JAMP to clearance of misfolded proteins.

The finding that RNF5 contributes to ERAD regulation by noncanonical ubiquitination of JAMP is consistent with the formation of Lys-63 ubiquitin chains by the E3 ligase POSH on the adaptor protein HERP (26). Ubiquitination by POSH alters HERP localization within the ER during the ER stress response and decreases (HERP association with proteasome subunits) (27). Intriguingly, both HERP and JAMP were shown to contribute to ERAD by recruitment of proteasome subunits.

Does RNF5 elicit two opposing functions in ERAD? The recognition that RNF5 also contributes to clearance of misfolded proteins as demonstrated for CFTRΔ508 (9, 10), and our data (Fig. 5) suggest this could be the case. Our data suggest that the contribution of RNF5 to degradation of ERAD substrates and control of the JAMP role in ERAD serves different phases of the ERAD process. This conclusion is based on the finding that RNF5 ubiquitination of JAMP occurs primarily prior to and following ER stress, as revealed from temporal analysis of the ER stress response. The timely effect of RNF5 on JAMP suggests a model (Fig. 7) in which RNF5 regulation of JAMP primarily occurs in the absence of ER stress. By contrast, the contribution of RNF5 to misfolded protein degradation occurs during ER stress or when misfolded proteins accumulate. RNF5 elicits diverse functions by cooperating with distinct E2-conjugating enzymes, resulting in ubiquitin chains of different topologies: Lys-48 for CFTR and Lys-63 for JAMP. The finding that Ubc7 can efficiently ubiquitinate JAMP is consistent with the notion that JAMP is also subject to proteasome-dependent degradation (Fig. 3, *a* and *b*), which is RNF5-independent (Fig. 2*b*). The latter may be mediated by other ER-associated ubiquitin ligases.

What determines the regulation of RNF5 of CFTR *versus* JAMP? Clearly the ability to mediate distinct ubiquitination of different substrates by the same E3 ligase suggests that other regulatory cues affect the activity of RNF5. For example, RNF5 reportedly requires an additional E3 ubiquitin ligase to recognize and promote degradation of misfolded CFTR protein, as shown for CHIP and gp78 (9, 10). Thus, RNF5 may associate with a third ligase to recruit Ubc13 to ubiquitinate JAMP. Alternatively, relative to ubiquitination of misfolded proteins, RNF5 ubiquitination of JAMP may require different organization of the ER membrane, enabling Ubc13 and contribution of other ER ligases. The relative position within the ER membrane may be also regulated by the topology of ubiquitin chains formed on RNF5/JAMP. Finally, distinct post-translational modification of RNF5 and/or substrates may control recruitment of a complex that mediates ubiquitination. Among the possibilities to consider is that the RNF5 complex with JAMP includes the stress-activated kinase JNK, which contributes to selective modification of complex components. Of note, following ER stress, Ubc13 levels are reduced, thereby limiting the availability of this E2 for Lys-63 ubiquitination of JAMP. It is of interest to determine the mechanism underlying regulation of Ubc13 levels following ER stress, which may affect the degree of noncanonical ubiquitination in response to select stress conditions.

Given the dual roles RNF5 plays in ERAD, one would expect impaired ERAD when RNF5 expression is deregulated. Would this be due to impaired degradation of misfolded proteins,

which would accumulate in the absence of RNF5, or to enhanced contribution of JAMP to the ERAD process, which should decrease levels of misfolded proteins? Based on the RNF5 transgenic mouse model in which misfolded proteins accumulate, we favor negative regulation of JAMP by RNF5 as a primary function, at least in the muscle IBM model. Consistent with this possibility is the observation that muscle samples from patients with IBM show elevated levels of RNF5 protein as well as high levels of ER stress markers (14). Elevated RNF5 expression is also reported in breast cancer and other tumor types and is associated with cytoskeletal organization, proliferation, and resistance to chemotherapy (28). Conversely, excessive degradation (hyper-ERAD) may result in severe pathological consequences, including rheumatoid arthritis, as was demonstrated for Hrd1 ligase (synviolin) (29, 30). That a tissue-specific effect may render RNF5 more important in targeting ubiquitination-dependent degradation of select misfolded proteins cannot be excluded.

Overall, this study reveals that the ER-anchored ubiquitin ligase RNF5 regulates JAMP via noncanonical ubiquitination, which limits the ability of JAMP to recruit proteasome and ERAD components, thereby decreasing its contribution to ERAD. Because RNF5 control of JAMP is primarily seen prior to and following ER stress, our findings suggest novel regulation of the ER stress response, in which ERAD activity is not required.

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REFERENCES

1. Brodsky, J. L. (2007) *Biochem. J.* **404,** 353–363
2. Hampton, R. Y. (2002) *Curr. Opin. Cell Biol.* **14,** 476–482
3. Ron, D., and Walter, P. (2007) *Nat. Rev. Mol. Cell Biol.* **8,** 519–529
4. Harding, H. P., Calfon, M., Urano, F., Novoa, I., and Ron, D. (2002) *Annu. Rev. Cell Dev. Biol.* **18,** 575–599
5. Wu, J., and Kaufman, R. J. (2006) *Cell Death Differ.* **13,** 374–384
6. Osborne, A. R., Rapoport, T. A., and van den Berg, B. (2005) *Annu. Rev. Cell Dev. Biol.* **21,** 529–550
7. Fang, S., Ferrone, M., Yang, C., Jensen, J. P., Tiwari, S., and Weissman, A. M. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 14422–14427
8. Kostova, Z., Tsai, Y. C., and Weissman, A. M. (2007) *Semin. Cell Dev. Biol.* **18,** 770–779
9. Morito, D., Hirao, K., Oda, Y., Hosokawa, N., Tokunaga, F., Cyr, D. M., Tanaka, K., Iwai, K., and Nagata, A. K. (2008) *Mol. Biol. Cell* **19,** 1328–1336
10. Younger, J. M., Chen, L., Ren, H. Y., Rosser, M. F., Turnbull, E. L., Fan, C. Y., Patterson, C., and Cyr, D. M. (2006) *Cell* **126,** 571–582
11. Broday, L., Kolotuev, I., Didier, C., Bhoumik, A., Podbilewicz, B., and Ronai, Z. (2004) *J. Cell Biol.* **165,** 857–867
12. Cox, E. A., and Hardin, J. (2004) *J. Cell Sci.* **117,** 1885–1897
13. Didier, C., Broday, L., Bhoumik, A., Israeli, S., Takahashi, S., Nakayama, K., Thomas, S. M., Turner, C. E., Henderson, S., Sabe, H., and Ronai, Z. (2003) *Mol. Cell. Biol.* **23,** 5331–5345
14. Delaunay, A., Bromberg, K. D., Hayashi, Y., Mirabella, M., Burch, D., Kirkwood, B., Serra, C., Malicdan, M. C., Mizisin, A. P., Morosetti, R., Broccolini, A., Guo, L. T., Jones, S. N., Lira, S. A., Puri, P. L., Shelton, G. D., and Ronai, Z. (2008) *PLoS ONE* **3,** e1609
15. Nogalska, A., Engel, W. K., McFerrin, J., Kokame, K., Komano, H., and Askanas, V. (2006) *J. Neurochem.* **96,** 1491–1499
16. Vattemi, G., Engel, W. K., McFerrin, J., and Askanas, V. (2004) *Am. J. Pathol.* **164,** 1–7
17. Engel, W. K., and Askanas, V. (2006) *Neurology* **66,** Suppl. 1, 20–29
18. Kadoya, T., Khurana, A., Tcherpakov, M., Bromberg, K. D., Didier, C., Broday, L., Asahara, T., Bhoumik, A., and Ronai, Z. (2005) *Mol. Cell. Biol.* **25,** 8619–8630
19. Tcherpakov, M., Broday, L., Delaunay, A., Kadoya, T., Khurana, A., Erdjument-Bromage, H., Tempst, P., Qiu, X. B., Demartino, G. N., and Ronai, Z. (2008) *Mol. Biol. Cell* **19,** 5019–5028
20. Wojcik, C., Rowicka, M., Kudlicki, A., Nowis, D., McConnell, E., Kujawa, M., and DeMartino, G. N. (2006) *Mol. Biol. Cell* **17,** 4606–4618
21. DeLaBarre, B., Christianson, J. C., Kopito, R. R., and Brunger, A. T. (2006) *Mol. Cell* **22,** 451–462
22. Habelhah, H., Frew, I. J., Laine, A., Janes, P. W., Relaix, F., Sassoon, D., Bowtell, D. D., and Ronai, Z. (2002) *EMBO J.* **21,** 5756–5765
23. Habelhah, H., Takahashi, S., Cho, S. G., Kadoya, T., Watanabe, T., and Ronai, Z. (2004) *EMBO J.* **23,** 322–332
24. Gomez, T. S., Hamann, M. J., McCarney, S., Savoy, D. N., Lubking, C. M., Heldebrant, M. P., Labno, C. M., McKean, D. J., McNiven, M. A., Burkhardt, J. K., and Billadeau, D. D. (2005) *Nat. Immunol.* **6,** 261–270
25. Chen, Z. J., and Fuchs, S. Y. (2004) *Cancer Biol. Ther.* **3,** 286–288
26. Tuvia, S., Taglicht, D., Erez, O., Alroy, I., Alchanati, I., Bicoviski, V., Dori-Bachash, M., Ben-Avraham, D., and Reiss, Y. (2007) *J. Cell Biol.* **177,** 51–61
27. Okuda-Shimizu, Y., and Hendershot, L. M. (2007) *Mol. Cell* **28,** 544–554
28. Bromberg, K. D., Kluger, H. M., Delaunay, A., Abbas, S., DiVito, K. A., Krajewski, S., and Ronai, Z. (2007) *Cancer Res.* **67,** 8172–8179
29. Yamasaki, S., Yagishita, N., Tsuchimochi, K., Kato, Y., Sasaki, T., Amano, T., Beppu, M., Aoki, H., Nakamura, H., Nishioka, K., and Nakajima, T. (2006) *Int. J. Mol. Med.* **18,** 113–117
30. Yamasaki, S., Yagishita, N., Tsuchimochi, K., Nishioka, K., and Nakajima, T. (2005) *Arthritis Res. Ther.* **7,** 181–186
31. Newton, K., Matsumoto, M. L., Wertz, I. E., Kirkpatrick, D. S., Lill, J. R., Tan, J., Dugger, D., Gordon, N., Sidhu, S. S., Fellouse, F. A., Komuves, L., French, D. M., Ferrando, R. E., Lam, C., Compaan, D., Yu, C., Bosanac, I., Hymowitz, S. G., Kelley, R. F., and Dixit, V. M. (2008) *Cell* **134,** 668–678