

REPORT

NEMF-mediated CAT tailing facilitates translocation-associated quality control

Amanda Ennis^{1*}, Lihui Wang^{1*}, Yue Xu¹, Layla Saidi¹, Xiaorong Wang², Clinton Yu², Sijung Yun¹, Lan Huang², and Yihong Ye¹

Ribosome stalling during co-translational translocation at the ER causes translocon clogging and impairs ER protein biogenesis. Mammalian cells resolve translocon clogging via a poorly characterized translocation-associated quality control (TAQC) process. Here, we combine a genome-wide CRISPR screen with live-cell imaging to dissect the molecular linchpin of TAQC. We show that TAQC substrates translated from mRNAs bearing a ribosome-stalling poly(A) sequence are degraded by lysosomes and the proteasome. By contrast, the degradation of defective nascent chains encoded by nonstop (NS) mRNAs involves an unconventional ER-associated protein degradation (ERAD) mechanism depending on ER-to-Golgi trafficking, KDEL-mediated substrate retrieval at the Golgi, and a tRNA-binding factor NEMF that appends an aggregation-prone carboxyl tail to stalled NS nascent chains. We propose that NEMF-mediated CAT tailing targets a subset of TAQC substrates via Golgi retrieval for ERAD, safeguarding ER homeostasis.

Introduction

Maintaining protein homeostasis is a critical cellular function involving various protein quality control (PQC) mechanisms. At the ER, a protein biogenesis site for secretory and membrane proteins, ER-associated protein degradation (ERAD) is a major PQC pathway. ERAD identifies and exports misfolded or incompletely assembled proteins to the cytoplasm for degradation by the ubiquitin–proteasome system (Bhattacharya and Qi, 2019; Christianson and Ye, 2014; Ruggiano et al., 2014). This process involves chaperones for substrate recognition, ubiquitin ligase-containing membrane complexes for protein retrotranslocation, and the conserved ATPase VCP/p97, which dislocates substrates from the ER membrane for cytosolic degradation by the proteasome (Vembar and Brodsky, 2008; Ye et al., 2001).

While significant progress has been made in understanding how ERAD removes misfolded proteins after their ER entry, less is known about how cells eliminate nascent chains stalled in the Sec61 translocon as a result of translation arrest during co-translational protein translocation (Wang and Ye, 2021). Translation arrest and/or ribosome stalling can be triggered by defective mRNAs, mRNAs with secondary structures, or difficult-to-translate sequences such as those coding the proline-rich domains of collagens (Wang et al., 2023; Yip and Shao, 2021). In

the cytoplasm, a ribosome-associated quality control (RQC) pathway resolves translation stalling, starting with the ubiquitination of small ribosomal proteins (Filbeck et al., 2022; Joazeiro, 2019). Ribosome-splitting factors then separate ribosomal subunits, exposing a binding site for the RQC complex consisting of the ubiquitin ligase LTN1 and nuclear export mediator factor (NEMF) (Shao et al., 2015). LTN1 ubiquitinates stalled nascent chains (Inada, 2020), while NEMF catalyzes CAT tailing, adding short peptide tails to these substrates in an mRNA-independent manner (Howard and Frost, 2021; Kostova et al., 2017). Although the role of LTN1 in RQC is well understood, the function of NEMF is less clear, particularly for dealing with ribosome stalling at the ER.

Ribosome stalling at the ER imposes a unique challenge, as translation-stalled nascent chains can clog the Sec61 translocon, inhibiting protein flow into the ER and secretion (Wang et al., 2020). Recent studies have shown that translation stalling at the ER activates a ubiquitin-like modifier 1 (UFM1) E3 ligase complex, which modifies the 60S ribosomal protein RPL26 with multiple single UFM1 moieties (Walczak et al., 2019; Wang et al., 2020). We further showed that RPL26 UFMylation engages a translocon-associated UFM1 sensor named SAYSD1, which facilitates the clearance of translocon “cloggers” by lysosomes

¹Laboratory of Molecular Biology, National Institute of Diabetes, Digestive, and Kidney Diseases, National Institutes of Health, Bethesda, MD, USA; ²Department of Physiology and Biophysics, University of California Irvine, Irvine, CA, USA.

*A. Ennis and L. Wang contributed equally to this paper. Correspondence to Yihong Ye: yihongy@mail.nih.gov

L. Wang's current affiliation is Innovent USA, Mountain View, CA, USA. S. Yun's current affiliation is Yottabiomed, LLC, Bethesda, MD, USA.

This is a work of the U.S. Government and is not subject to copyright protection in the United States. Foreign copyrights may apply. This article is distributed under the terms as described at <https://rupress.org/pages/terms102024/>.

(Wang et al., 2023; Wang and Ye, 2021). These findings define a translocation-associated quality control (TAQC) mechanism distinct from cytosolic RQC. However, a contradictory study concluded that the proteasome acts in conjunction with VCP/p97 to eliminate abnormal translation products at the ER via a mechanism similar to cytosolic RQC (Scavone et al., 2023).

In this study, we combine live-cell imaging with CRISPR-mediated genetic fingerprinting to define the fate of model translocon cloggers. Our results reveal an unexpected role for NEMF-mediated CAT tailing in TAQC, which unifies previous findings into a cohesive model. We propose that TAQC is a unique PQC mechanism bearing features of both RQC and ERAD.

Results and discussion

TAQC substrates can be degraded by both lysosomes and the proteasome

To resolve the discrepancy between our studies (Wang et al., 2020, 2023) and that of Kopito and colleagues (Scavone et al., 2023), we used live-cell confocal microscopy to reanalyze the fate of $_{ss}GFP$, which was shown to be a proteasome substrate (Scavone et al., 2023). $_{ss}GFP$ contains an ER-targeting signal sequence (SS) at the amino terminus of GFP, followed by 20 consecutive Lys residues, encoded by a poly(A) sequence known to stall ribosomes (Dimitrova et al., 2009; Juskiewicz and Hegde, 2017). When ribosomes stall, co-translational protein translocation also pauses, generating truncated polypeptides that clog the Sec61 translocon (Wang et al., 2020). We transfected U2OS cells with $_{ss}GFP$ and treated these cells with either ubiquitin-activating enzyme (E1) inhibitor (TAK-243), a VCP/p97 inhibitor (NMS-873), a lysosome inhibitor (Baf A1), a proteasome inhibitor (MG132), or a combination of Baf A1 and MG132. As expected, MG132 treatment stabilized $_{ss}GFP$ in a time-dependent manner, as did NMS-873 and TAK-243 (Fig. 1, A–D). Notably, MG132 and TAK-243 caused $_{ss}GFP$ to accumulate not only in the ER but also in large nuclear puncta in ~54 and ~40% of the transfected cells, respectively (Fig. 1 A, panels 2, 4). By contrast, NMS-873 only caused $_{ss}GFP$ to accumulate in the ER (panel 5). Co-staining with nucleolin antibodies confirmed the nuclear puncta as nucleoli (Fig. 1 E), suggesting that $_{ss}GFP$ is retrotranslocated; when its degradation in the cytosol is inhibited, $_{ss}GFP$ accumulates in nucleoli, likely because it contains a Lys-rich nucleolar localization sequence (Martin et al., 2015). Surprisingly, Baf A1 treatment also stabilized $_{ss}GFP$, as ~28% of $_{ss}GFP$ -expressing cells showed vesicle-like puncta, while ~22% cells contained nucleolus-like puncta and ~5% of cells had both (Fig. 1 A, panel 3). Co-immunostaining and live-cell imaging revealed extensive co-localization of $_{ss}GFP$ with either endogenous lysosomal protein LAMP1 or transfected LAMP1-mCherry (Fig. 1 F and Fig. S1 A). Although it is unclear why Baf A1 causes $_{ss}GFP$ to accumulate in nucleoli in some cells, our findings suggested that a fraction of $_{ss}GFP$ is degraded by lysosomes (Fig. 1 G). Interestingly, in cells co-treated with MG132 and Baf A1, we observed nucleolar and lysosomal accumulation of $_{ss}GFP$ in distinct populations of cells (~34% each), while only a small fraction of transfected cells (~6%) had both patterns (Fig. 1 A,

panel 6). These results suggest that cells use either the proteasome or lysosomes to degrade $_{ss}GFP$.

To further dissect the degradation mechanism of TAQC, we tested an additional SS-containing ribosome-stalling reporter, ER_{GFP_NS} . The mRNA-encoding ER_{GFP_NS} lacks any stop codons for translation termination, causing ribosome to stall (Fig. 1 G). When we treated HEK293T cells stably expressing ER_{GFP_NS} with Baf A1 or MG132, we found that MG132, but not Baf A1, increased GFP fluorescence (Fig. 1 H). Pulse-chase experiments further confirmed that ER_{GFP_NS} was short-lived, and its turnover was inhibited by MG132 but not by the lysosomal inhibitors chloroquine or Baf A1 (Fig. S1, B and C). Collectively, these results indicate that ER_{GFP_NS} degradation is mediated entirely by the proteasome (Fig. 1 G).

ER_{GFP_NS} is degraded by an ERAD-like mechanism involving SAYSD1 and the TRAPP complex

The observed proteasomal degradation of ER_{GFP_NS} is surprising because, like previously reported TAQC substrates ER_{GFP_K20} and $_{ss}GFP$ (Scavone et al., 2023; Wang et al., 2023), the degradation of ER_{GFP_NS} also requires UFM1 (Wang et al., 2023). We therefore combined cell fractionation with inhibitor treatment and UFM1 knockdown to dissect how UFM1 cooperates with the proteasome to promote ER_{GFP_NS} degradation. Consistent with UFM1 being a positive TAQC facilitator, immunoblotting detected ER_{GFP_NS} accumulation in UFM1-depleted cells in both full-length (~45 kDa) and an SDS-resistant high molecular weight (HMW) form (Fig. 2 A, lane 2). The HMW ER_{GFP_NS} “smear” was not apparent in proteasome inhibitor-treated cells, excluding ubiquitination as the main cause of this molecular weight shift. Instead, MG132 treatment caused accumulation of mostly full-length and a cleaved ER_{GFP_NS} species, each containing a fast- and a slow-migrating band on the blot (lane 3 versus 1). Biochemical fractionation showed that ER-enriched microsomes from MG132-treated cells had all four ER_{GFP_NS} bands, but the corresponding cytosolic fraction contained only the fast-migrating band for each species (Fig. 2 B, lane 4 versus 3). When membrane fractions from MG132-exposed cells were treated with Endo H, the slow-migrating ER_{GFP_NS} forms were converted to the corresponding fast-migrating species (Lane 6 versus 5), suggesting that the former was caused by N-glycosylation. Collectively, these results suggest that ER_{GFP_NS} is retrotranslocated from the ER and deglycosylated similarly to previously reported glyco-ERAD substrates (Shamu et al., 1999). Consistent with this notion, the VCP/p97 inhibitor NMS-873 only caused ER_{GFP_NS} to accumulate in the glycosylated forms (Fig. 2 A, lane 5). Knockdown of UFM1 in MG132- or NMS-873-treated cells reduced both deglycosylated and cleaved ER_{GFP_NS} (Fig. 2 A, lanes 4, 6 versus 3, 5), suggesting that UFMylation acts upstream of retrotranslocation.

Our previous study demonstrated that the lysosomal transport of ER_{GFP_K20} requires a translocon-associated, ribosome- and UFM1-binding protein SAYSD1 and the trafficking protein particle (TRAPP) complex (Wang et al., 2023), the latter is a known Rab1 modulator regulating the ER-to-Golgi trafficking (Barrowman et al., 2010). We therefore tested whether these factors also regulate ER_{GFP_NS} trafficking and turnover. First,

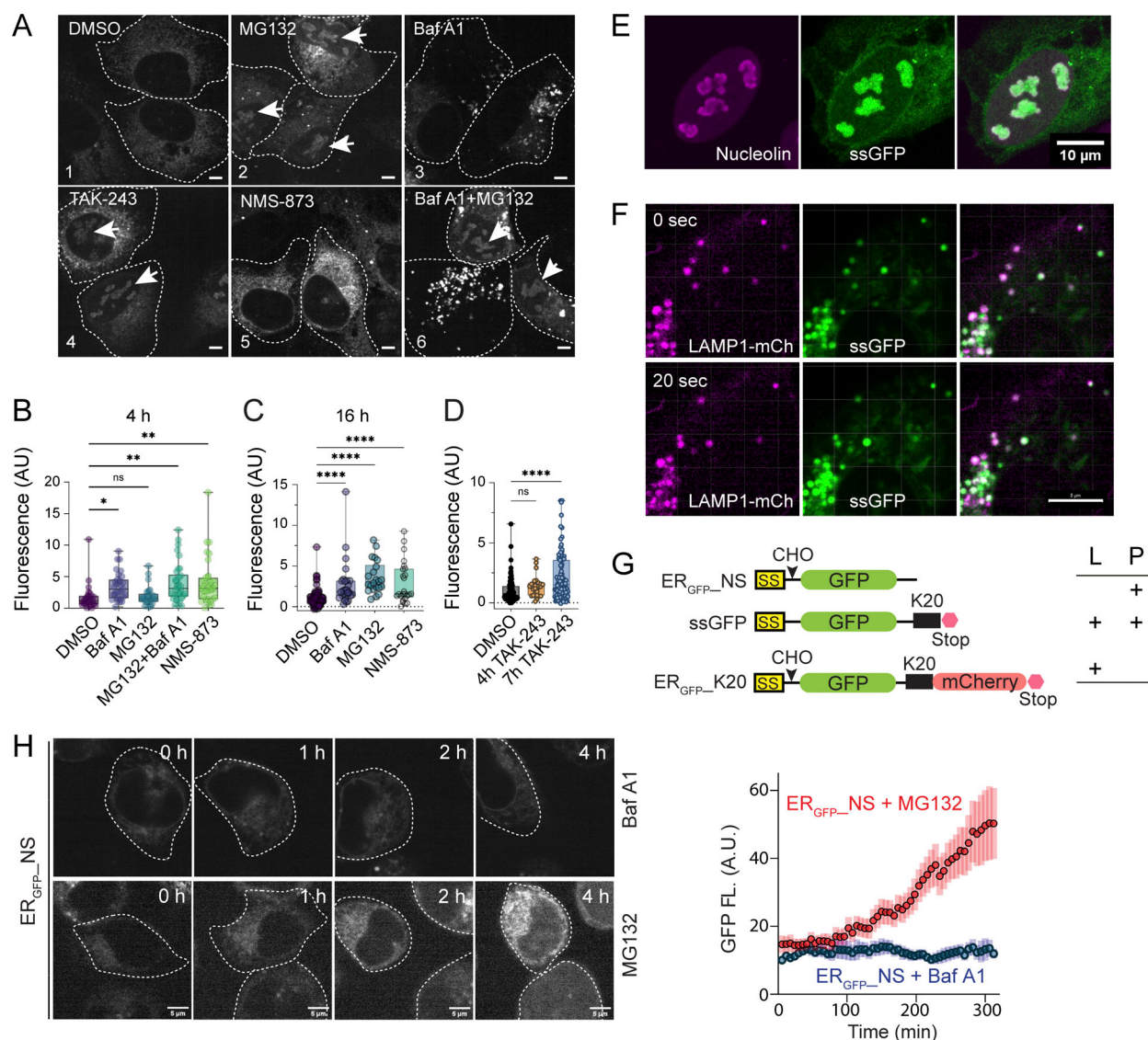


Figure 1. TAQC substrates can be degraded by both the proteasome and lysosomes. (A) U2OS cells transfected with ssGFP were treated with the indicated inhibitors for 4 h and imaged. Arrowheads indicate nucleolar localization of ssGFP. Scale bars, 5 μ m. **(B–D)** Quantification of GFP fluorescence in ssGFP-transfected cells treated with the indicated inhibitors. Shown is box and whiskers plot with all points indicated. The boxes show the upper quartile, the median, and the lower quartile. ns, not significant; * $P < 0.05$; ** $P < 0.01$; **** $P < 0.0001$ by one-way ANOVA's Dunnett's multiple comparison test. $n > 20$ cells in randomly selected fields from two independent experiments. **(E)** ssGFP accumulates in nucleoli in MG132-treated cells. U2OS cells expressing ssGFP were treated with MG132 and stained with anti-nucleolin antibodies (magenta). Scale bar, 10 μ m. **(F)** ssGFP accumulates in LAMP1-mCherry (mCh)-positive lysosomes in Baf A1-treated cells. Shown are two time points from a live-cell imaging experiment. Scale bar, 8 μ m. **(G)** Schematic illustration of the translation-stalled ER reporters and their degradation pathways. L: lysosome; P: proteasome. **(H)** Left panels, ER_{GFP}-NS-transfected HEK293T cells were treated with Baf A1 (200 nM) or MG132 (20 μ M) and imaged continuously for 6 h. Scale bars, 5 μ m. The graph shows the quantification of the experiment. Error bars, SEM. $n = 6$ cells for each condition.

we used SAYSD1-specific siRNA to knock down SAYSD1 in ER_{GFP}-NS-expressing HEK293T cells. Confocal microscopy detected a significant accumulation of ER_{GFP}-NS in SAYSD1-depleted cells (Fig. 2 C). Co-immunostaining showed that ER_{GFP}-NS accumulated in SAYSD1 knockdown cells was largely co-localized with the ER marker calreticulin (Fig. S2 A). We next tested whether ER_{GFP}-NS interacts with SAYSD1 by co-immunoprecipitation. To this end, we transfected WT and UFM1 knockout cells with either an empty vector or an ER_{GFP}-NS-expressing construct. Immunoprecipitation with GFP antibodies pulled down ER_{GFP}-NS together with endogenous

SAYSD1 and Sec61 β from WT cells (Fig. 2 D, lane 4). By contrast, in UFM1 knockout cells, although we detected more ER_{GFP}-NS and Sec61 β in the precipitated samples, less SAYSD1 was co-precipitated (lane 8 versus 4). No SAYSD1 was detected when immunoprecipitation was performed using empty vector-transfected cells (lane 3). These results suggest that, similar to ER_{GFP}-K20 (Wang et al., 2023), ER_{GFP}-NS interacts with SAYSD1 in a UFMylation-dependent manner.

To determine whether the interaction between ER_{GFP}-NS and SAYSD1 depends on RPL26 UFMylation, we repeated the co-immunoprecipitation experiment using a CRISPR-engineered

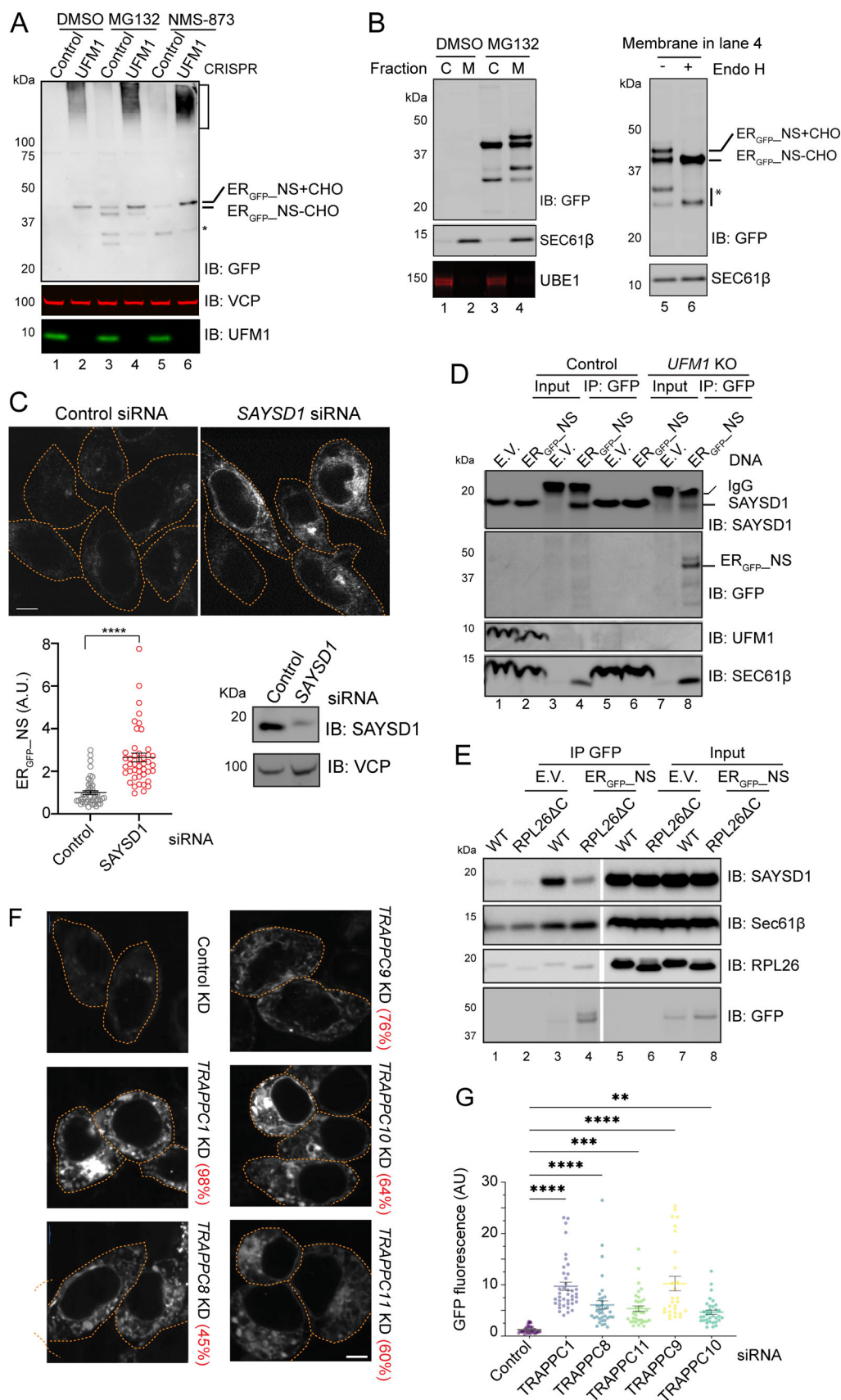


Figure 2. **Ribosome UFMylation, SAYSD1, and the TRAPP complex facilitate ER_{GFP-NS} degradation.** (A) Control or UFM1 knockout HEK293T cells were transfected with ER_{GFP-NS} and treated with the indicated inhibitors for 6 h. Cell extracts were analyzed by immunoblotting (IB). +CHO, glycosylated; -CHO,

deglycosylated. **(B)** ER_{GFP}-NS-expressing cells treated as indicated were fractionated into a membrane (M) and cytosol (C) fraction before immunoblotting (left panel). Right panel, the membrane fraction used in lane 4 was treated with Endo H and then analyzed by immunoblotting. *, cleaved ER_{GFP}-NS fragments. **(C)** Top panels, HEK293T cells were transfected with the indicated siRNAs together with ER_{GFP}-NS. Cells were imaged 48 h after transfection. A fraction of the cells was analyzed by immunoblotting to confirm the knockdown efficiency (bottom right). The graph shows the quantification result from three independent experiments. $N > 40$ cells. **** $P < 0.0001$ by unpaired Student's t test. **(D)** Control or UFM1 KO cells were transfected with either empty vector (EV) or ER_{GFP}-NS. Cells were lysed in CHAPS buffer. The lysates were subject to immunoprecipitation with GFP antibodies before immunoblotting. Note that the detergent in input samples affects the mobility of low molecular weight proteins. **(E)** As in D, except that RPL26ΔC and an isogenic WT line were used. **(F)** HEK293T cells transfected with the indicated siRNAs and ER_{GFP}-NS were analyzed by confocal microscopy. The numbers indicate the knockdown efficiency determined by qRT-PCR averaged from three independent experiments. **(G)** Quantification of experiments represented in F. At least 30 randomly selected cells from two independent experiments were analyzed. Error bars, SEM. ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$ by one-way ANOVA's Dunnett's multiple comparison test. IP, immunoprecipitated. Scale bars in C and F, 5 μm. Source data are available for this figure: SourceData F2.

knock-in cell line that has the RPL26 carboxyl-terminal UFMylation sites removed (RPL25ΔC) (Wang et al., 2023). Like in UFM1 knockout cells, GFP antibodies precipitated more ER_{GFP}-NS from RPL26ΔC cells, but less SAYSD1 was co-precipitated (Fig. 2 E, lane 4 versus 3), suggesting that RPL26 UFMylation enhances ER_{GFP}-NS's interaction with SAYSD1. By contrast, the interactions of ER_{GFP}-NS with the Sec61 translocon and ribosome were not affected in RPL26ΔC cells. Together, these findings implicate SAYSD1 as a regulator that engages ER_{GFP}-NS downstream of RPL26 UFMylation to facilitate its degradation.

To test whether ER_{GFP}-NS degradation requires the TRAPP complex, we used siRNA to knock down several TRAPP complex components in ER_{GFP}-NS cells. Quantitative RT-PCR (qRT-PCR) confirmed the knockdown efficiency, ranging from 45 to 98% (Fig. 2 F). Fluorescence confocal microscopy showed significant upregulation of ER_{GFP}-NS in cells depleted of TRAPPC1, TRAPPC8, TRAPPC9, TRAPPC10, or TRAPPC11 (Fig. 2, F and G). Co-immunostaining showed that in cells depleted of TRAPPC8, ER_{GFP}-NS mainly accumulated in a perinuclear region overlapping with the ER marker calreticulin (Fig. S2 B). Thus, TRAPPC-mediated ER-to-Golgi trafficking also facilitates ER_{GFP}-NS degradation.

ER_{GFP}-NS degradation requires an ER-retrieval pathway and ERAD

To define the genetic requirement of ER_{GFP}-NS degradation, we conducted an unbiased genome-wide CRISPR screen using HEK293T cells stably expressing ER_{GFP}-NS. To this end, we targeted the ~20,000 human genes, each with six single gRNAs (sgRNAs), expressed from a lentivirus-based CRISPR-Cas9 library (Sanjana et al., 2014). The transduced cells were separated into a GFP-high (~10%) and the remaining (~90%) population by flow cytometry. We then used PCR amplification and high-throughput sequencing to identify sgDNAs enriched in the GFP-high population. Statistical analysis revealed 832 genes whose inactivation increased ER_{GFP}-NS significantly (Fig. 3 A). We compared this list to genes identified previously in a similar screen using ER_{GFP}-K20 as the reporter (Wang et al., 2023). Since these two substrates are degraded by different mechanisms, most identified genes did not overlap, as expected. Nevertheless, 88 genes (~5%), when inactivated, increased GFP fluorescence of both ER_{GFP}-K20 and ER_{GFP}-NS. STRING-based protein network analyses showed that most common "hits" were associated with RNA catabolism, which likely affected these reporters via changing the mRNA abundance. Notably, the

overlapping gene list includes several UFMylation components and ER-to-Golgi trafficking regulators, such as components of the TRAPP complex (Fig. 3 B and Fig. S3), further validating the involvement of UFMylation and ER-to-Golgi trafficking in ER_{GFP}-NS degradation. Our screen did not uncover SAYSD1 and several other factors implicated in ER_{GFP}-K20 degradation (e.g., some TRAPPC subunits, LTN1, and NEMF), suggesting that the screen is not saturated.

Next, we used STRING-based analysis to identify functional networks associated only with the degradation of ER_{GFP}-NS but not ER_{GFP}-K20. This approach identified one major network—ERAD—and several smaller ones (Fig. 3 C). While the role of these small networks in ER_{GFP}-NS regulation awaits further characterization, siRNA-mediated knockdown of Hrd1/SYVN1 or VCP/p97, two well-established ERAD regulator (Bhattacharya and Qi, 2019), significantly increased the ER_{GFP}-NS level in cells (Fig. 3, D and E), confirming the role of ERAD in ER_{GFP}-NS degradation.

At first glance, the degradation of ER_{GFP}-NS by ERAD appears inconsistent with the involvement of UFMylation, SAYSD1, and TRAPP-dependent ER-to-Golgi trafficking in this pathway. However, further examination of the gene networks specific for ER_{GFP}-NS degradation identified components of the KDEL-dependent ER-retrieval pathway, including KDELR2, COPB1, and RAB1B (Fig. 3 C). This finding raised the possibility that ER_{GFP}-NS is exported to the Golgi and then retrieved back to the ER before ERAD. Consistent with this view, knockdown of KDELR2, COPB1, RAB1B, and TMED3 in ER_{GFP}-NS cells not only increased ER_{GFP}-NS fluorescence but also caused ER_{GFP}-NS to accumulate in a perinuclear region, co-localizing with the Golgi marker, cholera toxin B (Fig. 3, F and G). Altogether, our results suggest that following TRAPP-dependent transport to the Golgi, ER_{GFP}-NS is retrieved back to the ER before undergoing retro-translocation and ERAD.

NEMF-dependent CATylation is involved in the degradation of ER_{GFP}-NS

Previous studies showed that the degradation of ER_{GFP}-K20 and ssGFP involves LTN1 and NEMF (Scavone et al., 2023; Wang et al., 2023). LTN1 catalyzes ubiquitination, while NEMF mediates CATylation of translation-stalled proteins (Inada, 2020). To explore whether these factors regulate ER_{GFP}-NS degradation, we transfected into U2OS cells ER_{GFP}-NS together with siRNAs targeting LTN1 or NEMF. Immunoblotting showed that knockdown of LTN1 by ~50% also downregulated NEMF1 to a similar

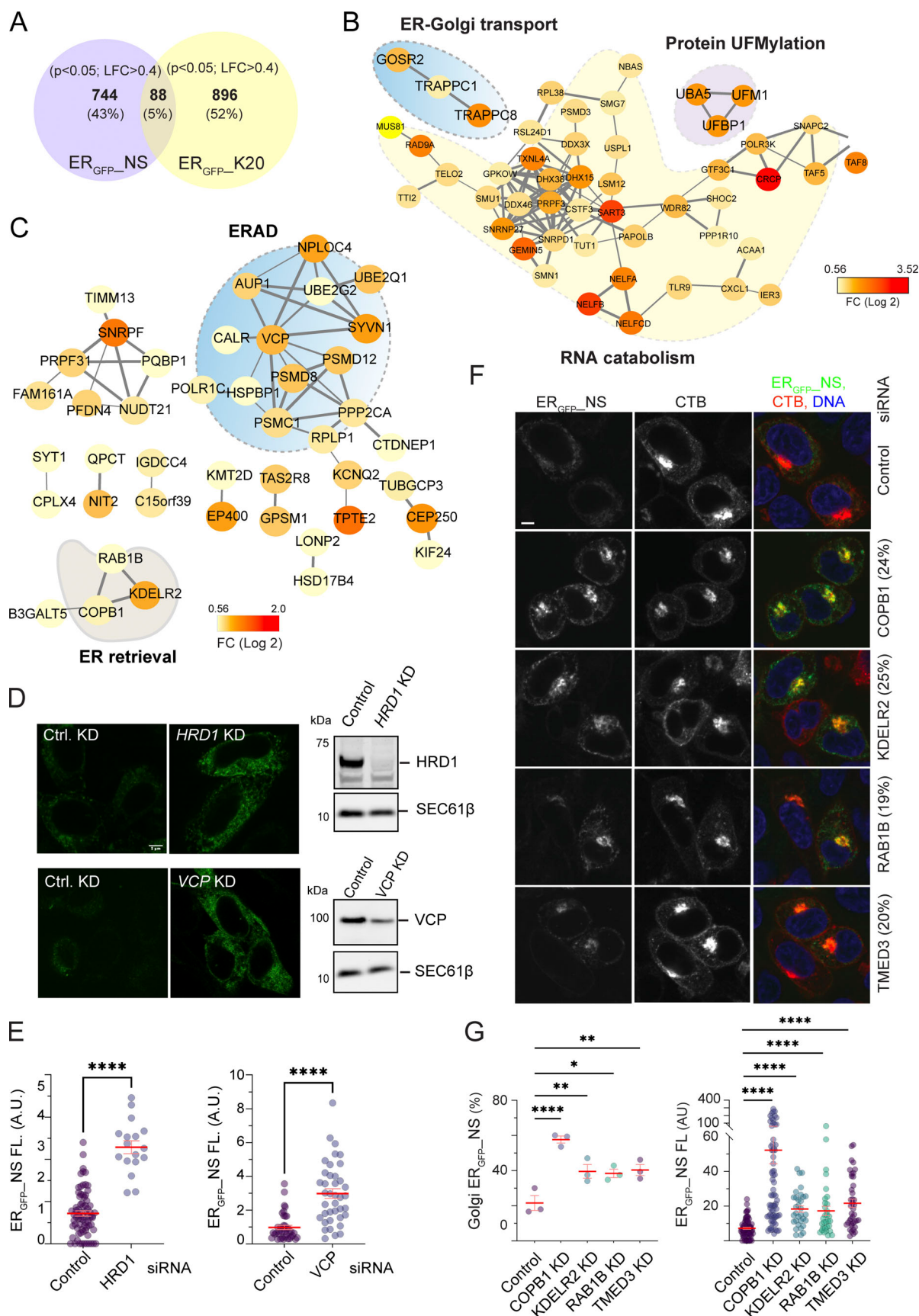


Figure 3. **A** CRISPR screen reveals the genetic requirement for the ER_{GFP}-NS degradation pathway. **(A)** A Venn diagram shows the number of genes whose inactivation increased ER_{GFP}-NS and ER_{GFP}-K20, respectively. LFC, log fold change. **(B)** A STRING network analysis reveals gene networks that affect

the steady-state levels for both ER_{GFP}_NS and ER_{GFP}_K20. **(C)** STRING networks affecting only the level of ER_{GFP}_NS. Node color in B and C indicates fold enrichment in GFP-high populations. **(D)** HEK293T cells were transfected with control, Hrd1, or VCP/p97 siRNA together with ER_{GFP}_NS. Cells were imaged by fluorescence confocal microscopy to measure the ER_{GFP}_NS levels (left) or by immunoblotting (right) to confirm gene knockdown. Scale bar, 5 μ m. **(E)** Quantification of two independent experiments as shown in D. Error bars, SEM, *****P* < 0.0001 by unpaired Student's *t* test. **(F)** HEK293T cells were transfected with the indicated siRNAs followed by ER_{GFP}_NS. Cells were stained by Alexa⁵⁹⁴-labeled cholera toxin subunit B to identify the Golgi. Scale bar, 5 μ m. **(G)** Quantification of the percentage of transfected cells showing a Golgi localization of ER_{GFP}_NS (left) or the total fluorescence intensity of ER_{GFP}_NS (right) from three independent experiments. Error bars, SEM. **P* < 0.05; ***P* < 0.01; *****P* < 0.0001 by one-way ANOVA's Dunnett's multiple comparison test. Source data are available for this figure: SourceData F3.

level, whereas knockdown of NEMF did not affect LTN1 (Fig. 4 A). Confocal microscopy showed that knockdown of NEMF or LTN1 caused approximately fivefold upregulation of ER_{GFP}_NS in cells (Fig. 4, B and C). Immunoblotting further confirmed that knockdown of *LTN1* or *NEMF* increased the steady-state ER_{GFP}_NS level, similarly to knockdown of *UFM1*; this phenotype was observed regardless of whether cells were treated with a low concentration of anisomycin, which induces ribosome stalling and RPL26 UFMylation (Fig. 4, D and E). Noticeably, *NEMF* or *LTN1* knockdown did not cause ER_{GFP}_NS to accumulate in the SDS-resistant HMW form, as it was seen in *UFM1* knockdown cells (Fig. 4 D, lanes 3, 4 versus lanes 5–8). However, when *NEMF* and *UFM1* were knocked down simultaneously, no HMW smear was observed (Fig. 4 D, lanes 9, 10 versus 3, 4; Fig. 4 F). Given the well-established function of NEMF in CATylation of translation-stalled polypeptides (Lyumkis et al., 2014; Shen et al., 2015; Tesina et al., 2023), the HMW ER_{GFP}_NS species likely results from NEMF-mediated CATylation, which accumulates when its degradation is inhibited in *UFM1*-depleted cells. Since CATylation usually appends small tags of <5 kDa to stalled nascent chains, as reported for ssGFP and cytosolic RQC substrates (Mizuno et al., 2021; Scavone et al., 2023; Shen et al., 2015; Thrün et al., 2021), the larger molecular weight shift seen with ER_{GFP}_NS suggested that the tail appended to ER_{GFP}_NS might have a sequence composition more prone to aggregation.

To further prove that the HMW ER_{GFP}_NS species is a CATylation product, we generated an ER_{GFP}_NS variant that has a tobacco etch virus (TEV nuclear inclusion—an endopeptidase) protease site ~60 amino acids upstream of the stalling site (ER_{GFP}_TEV_NS) (Fig. 4 G). Like ER_{GFP}_NS, the TEV-bearing NS variant was also stabilized by MG132, but not by Baf A1. When ER_{GFP}_TEV_NS was immunoprecipitated from *UFM1* knockdown cells and treated with TEV, its carboxyl terminus was cleaved (Fig. 4 H, middle panel), which also caused the disappearance of the HMW ER_{GFP}_TEV_NS species (top panel), suggesting that the formation of this HMW species depends on the carboxyl terminus of ER_{GFP}_TEV_NS.

To see whether the carboxyl terminus of ER_{GFP}_TEV_NS is required for its degradation, we co-expressed ER_{GFP}_TEV_NS with ER-targeted TEV protease (mCh-ER_{TEV}) in U2OS cells and used confocal microscopy to compare the level of ER_{GFP}_TEV_NS in mCh-ER_{TEV}-positive cells to that in mCh-ER_{TEV}-negative cells in the same field. If the carboxyl terminus is essential for ER_{GFP}_TEV_NS degradation, TEV-positive cells should accumulate ER_{GFP}_TEV_NS to a higher level than TEV-negative cells, which was indeed observed (Fig. 4, I and J). By contrast, mCh-ER_{TEV} expression did not affect ER_{GFP}_NS that lacks the TEV site.

Collectively, these results suggest that ER_{GFP}_NS is subjected to NEMF-mediated CATylation, which appends a CAT tail to facilitate its degradation.

Our study has revealed two distinct degradation pathways for nascent chains that clog the Sec61 translocon in TAQC, which resolves a major discrepancy in the field. We show that “translocon cloggers” released into the ER lumen can be transported to lysosomes or targeted for proteasomal degradation. In both cases, they use a mechanism that depends on RPL26 UFMylation, SAYSD1, and the TRAPP complex to traffic out of the ER. Once arriving at the Golgi, some substrates, as exemplified by ER_{GFP}_NS, use a KDEL-dependent retrieval pathway to return to the ER for subsequent elimination by ERAD. Others (e.g., ER_{GFP}_K20) continue to reach lysosomes (Fig. 4 K). Although we cannot exclude that some cloggers in an early phase of protein translocation (those with only a small portion inserted into the ER) might “slip” back into the cytoplasm and be degraded by a mechanism akin to cytosolic RQC (Scavone et al., 2023), our results are consistent with the finding that defective translocation products are mostly released into the ER lumen before being targeted by PQC mechanisms (Arakawa et al., 2016).

Our study also demonstrates a critical function for NEMF in TAQC. NEMF and its yeast homolog Rqc2 catalyze a unique co-translational modification to facilitate substrate ubiquitination by ribosome-associated LTN1 or other cytosolic ubiquitin ligases (Sitron and Brandman, 2019; Thrün et al., 2021). However, whether CAT tailing occurs and its roles in TAQC have been unclear since substrates released into the ER lumen are inaccessible by these cytosolic ubiquitin ligases. In this regard, although knockdown of LTN1 and NEMF both inhibits the degradation of model TAQC substrates (Crowder et al., 2015; Scavone et al., 2023; Wang et al., 2023), the LTN1's contribution to TAQC may be limited to NEMF stabilization (Fig. 4 A) (Lv et al., 2024). Because CAT tail added to ER_{GFP}_NS is more aggregation prone than those on ER_{GFP}_K20 or ssGFP, and because these substrates are triaged differently, we propose that NEMF-mediated CAT tailing may have a sorting function in mammalian TAQC. Future studies are required to determine whether NEMF can specify distinct CAT tails for TAQC substrates and how this process is linked to distinct triaging fates in TAQC.

Materials and methods

Chemicals, plasmids, and antibodies

Bafilomycin A1, NMS-873, and MG132 were purchased from LC Laboratories, Xcess Biosciences, and Millipore, respectively. TAK-243 was from Selleck Chemicals. Anisomycin was purchased from Sigma-Aldrich. Bortezomib was from Takeda

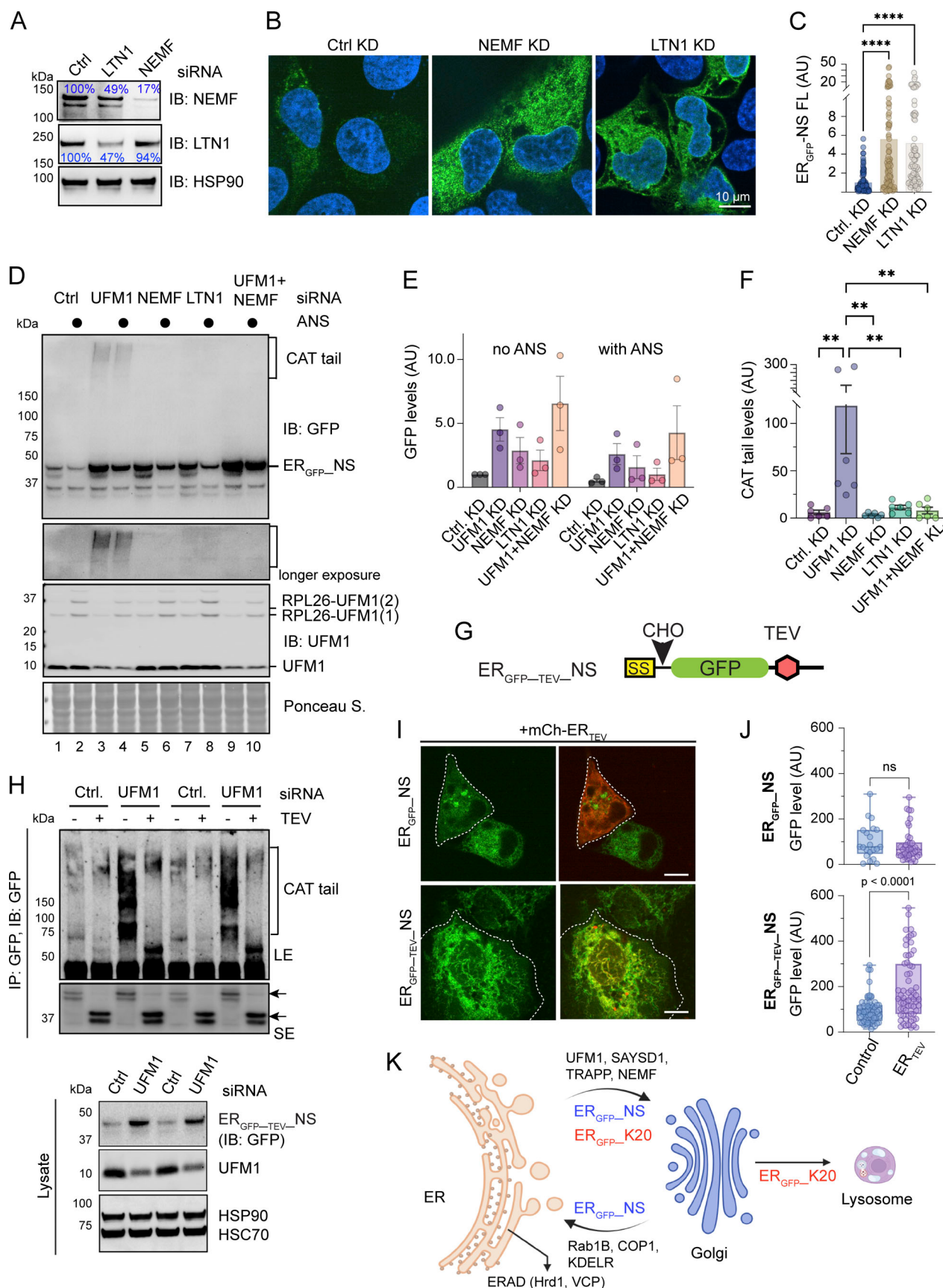


Figure 4. **NEMF1 facilitates ER_{GFP}_NS degradation via CAT tailing.** (A) U2OS cells transfected with the indicated siRNA together with ER_{GFP}_NS were lysed and analyzed by immunoblotting to verify knockdown efficiency (indicated by the numbers). (B) U2OS cells transfected with the indicated siRNAs and

ER_{GFP}-NS were stained with Hoechst and imaged. Scale bar, 10 μ m. **(C)** Quantification of two independent experiments as shown in B. Error bars, SEM, ****P = 0.0001 by one-way ANOVA's Dunnett's multiple comparison test. **(D)** HEK293T cells transfected with the indicated siRNAs and ER_{GFP}-NS were treated with anisomycin (ANS, 200 nM) for 1 h. Cell lysates were analyzed by immunoblotting. **(E)** Quantification of ER_{GFP}-NS levels in three independent experiments represented by D. Error bars, SEM. **(F)** Quantification of the HMW smear level in the indicated knockdown cells. Error bars, SEM, **P < 0.01 by one-way ANOVA's Dunnett's multiple comparison test. **(G)** Schematic of the ER_{GFP}-TEV-NS construct. **(H)** ER_{GFP}-TEV-NS was immunoprecipitated (IP) from control or UFM1 knockdown HEK293T cells and treated with TEV protease (+) (0.01 μ g/ μ l, 3 h) or mock treated (-) before immunoblotting (IB). Top panels show long (LE) and short (SE) exposures from two independent experiments. Lower panels show IB analyses of a fraction of the corresponding lysates. **(I)** Representative images of U2OS cells transfected with mCherry-tagged ER_{TEV} (mCh-ER_{TEV}) together with ER_{GFP}-TEV-NS or ER_{GFP}-NS. Scale bar, 10 μ m. **(J)** Quantification of GFP fluorescence in individual cells as shown in I. P values were by unpaired Student's t test, n = 2 independent experiments. ns, not significant. **(K)** A model of distinct TAQC branches. Source data are available for this figure: SourceData F4.

Pharmaceuticals. Cholera toxin subunit B (CF 594 conjugated) was from Biotium.

The pER_{GFP}-K20 and pER_{GFP}-NS plasmids were reported previously (Wang et al., 2020). We purchased the plasmid expressing an ER-localized hook protein from Addgene (#65308; li-Str_Neomycin). To generate various model substrates with artificial CAT tails, we used restriction enzymes BamHI and XbaI to cut pER_{GFP}-K20 (#133861; Addgene plasmid), removing most of the sequence downstream of GFP-3xFLAG. Various CAT tail-encoding sequences (listed below) were synthesized by IDT and PCR-amplified using the corresponding primer pairs. PCR products were digested with BamHI and XbaI. The digested products were ligated into digested pER_{GFP}-K20. To make pGFP_{ER}-ext, a stop codon was introduced at the site to include a short nucleotide (encoding NH2-AGSPGTPSGTNVGS-COOH) in the ORF downstream of 3xFLAG by site-directed mutagenesis. To make pGFP_{ER}, a stop codon was introduced right after the 3xFLAG-coding sequence. ER_{GFP}-TEV-NS was generated by inserting a DNA fragment coding the TEV site (ENLYFQ/S), 177-bp upstream of the predicted RNA termination site using PCR-based mutagenesis.

Inhibitor treatments

To test the effect of several inhibitors on the expression and trafficking of ssGFP and model substrates with artificial CAT tails, U2OS were seeded at 25,000 cells/well on an 8-well chamber. After 24 h, cells were co-transfected with 250 ng ssGFP plus 50 ng mCh-Lamp1 (to identify localization of ssGFP expression) or with 200 ng CAT tail-containing model substrates using Invitrogen's Lipofectamine 2000 transfection reagent. 24 h later, cells were treated for ~6 h with either 200 nM Baf A1, 20 μ M MG132, or 1 μ M bortezomib and imaged by live-cell imaging. To cause partial translation arrest, cells were treated with 200 nM anisomycin for 1 h before analysis. To test the degradation pathway for ssGFP, cells transfected with ssGFP for 24 h were treated with 200 nM Baf A1, 20 μ M MG132, 1 μ M bortezomib, 5 μ M NMS-873, or 10 μ M TAK243 before imaging.

Genome-wide CRISPR/Cas9 knockout screen

A genome-wide CRISPR/Cas9 knockout screen was performed as described previously (Wang et al., 2023). Briefly, the Geckov2 library (#1000000048; Addgene) was amplified according to the online protocol (Sanjana et al., 2014), and the complexity of the library was determined by high-throughput sequencing. Lentiviruses expressing the sgRNA library and Cas9 were used for spin infection as follows: 60 million ER_{GFP}-NS-expressing stable

HEK293T cells supplemented with 8 μ g/ml polybrene (Sigma-Aldrich) were seeded into two 12-well plates at a density of 3 million cells/well. Concentrated GeCKO v2 lentivirus was added to each well at a MOI of 0.3. Cells were spun at 1,000 g at room temperature for 2 h, followed by incubation at 37°C in a humidified incubator for 1 h. After medium removal, fresh medium was added, and cells were incubated for 48 h before puromycin selection (0.3 μ g/ml). The transduced cells were subcultured in medium supplemented with puromycin every 2 days for a total of 8 days, and 80 million cells were maintained for each passage. After puromycin treatment, cells were recovered in a medium lacking puromycin for 24 h before sorting. In total, 60 million cells were sorted into GFP-high (1% of total cells) or GFP-low (80% of total cells) cell populations by a FACS AriaII cell sorter. Genomic DNA was extracted from each cell population using a QIAGEN Blood Maxi kit (for GFP low cells) or QIAGEN Blood Midi kit (for GFP high cells) according to the manufacturer's instructions. sgRNAs were amplified from genomic DNA samples using the Hercules II Fusion DNA polymerase (Agilent Technologies) in two PCR steps as follows. In the first PCR, the genomic region containing sgRNAs was amplified from 130 μ g total DNA using the following primers: forward: 5'-AATGGA CTATCATATGCTTACCGTAACCTTGAAAGTATTTTCG-3'; reverse: 5'-CTTTAGTTTGTATGTCTGTTGCTATTATGTCTACTATTCTTT CC-3'. In total, 13 PCR reactions were performed in parallel, with 10 μ g genomic DNA in each reaction using Hercules II Fusion DNA polymerase (Agilent) for 18 cycles, and the resulting PCR products were combined. In the second step of PCR, 5 μ l of the first PCR product was used in a 100 μ l reaction volume, and 24 PCR cycles were used. The primers used for the second PCR include staggered sequences of variable lengths and a 6-bp barcode for multiplexing of different biological samples. The second step PCR primers used are forward: 5'-AATGATACGGCG ACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCG ATCT-3' (1-9-bp variable length sequence) (6-bp barcode) 5'-TCTTGTGGAAAGGACGAAACACCG-3'; reverse: 5'-CAAGCA GAAGACGGCATACGAGATGTGACTGGAGTTCAGACGTGTGCT CTTCGATCTTCTACTATTCTTTCCCTGCACTGT-3'. The PCR products were gel extracted, quantified, and sequenced using a NovaSeq sequencer (Illumina) by the National Heart, Lung, and Blood Institute (NHLBI) DNA Sequencing and Genomics Core. sgRNA sequences were obtained per sample by extracting 20 bps followed by the index sequence of "5'-TTGTGGAAAGGACGA AACACCG-3'" on the de-multiplexed FASTQ files from Illumina's NGS sequencer using the Cutadapt software, version 2.8 (<https://doi.org/10.14806/ej.17.1.200>, Martin, 2011). FASTQC,

version 0.11.9, was used to assess the sequencing quality (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). MAGeCK, version 0.5.9, was used to quantify and to identify differentially expressed sgRNAs. The MAGeCK count command was run on the merged two-half libraries of A and B to quantify. Differentially expressed sgRNAs with statistical significance were determined by running the MAGeCK test command in paired mode. Genes were ranked based on the number of unique sgRNAs enriched in the GFP-high population versus the GFP-low population. Data were derived from two biological repeats.

Cells and siRNA transfections

For all experiments, HEK293T and U2OS cells (ATCC) were cultured in DMEM (Corning) containing 10% FBS and antibiotics (penicillin/streptomycin, 10 U/ml) at 37°C in a 5% CO₂ humidified incubator. To test the effect of NEMF and UFM1 knockdowns on ER_{GFP-NS} expression by microscopy, U2OS cells were seeded at 25,000 cells/well on an 8-well chamber (Ibidi). 24 h after seeding, cells were transfected with 4 pmol of siRNAs targeting genes specified in the figure legends using Invitrogen RNAiMax transfection reagent. 24 h later, cells were transfected with 300 ng ER_{GFP-NS} with Invitrogen Lipofectamine 2000 transfection reagent. Cells were incubated with fresh media for 24 h before fixation and imaging. A similar protocol was used to knock down COPB1, KDELR2, RAB1B, and TMED3 in HEK293T cells to check the role of these genes in ER_{GFP-NS} trafficking by fluorescence microscopy. In some experiments, a Cy3-labeled negative control siRNA was included to verify transfection efficiency.

To test the effect of NEMF and UFM1 knockdowns on ER_{GFP-NS} protein level by immunoblotting, HEK293T cells were seeded on a 6-well plate at 500,000 cells/well. Plates were pre-coated with poly-D-lysine to assist with cell adherence to the well. 24 h after seeding, cells were transfected with 120 pmol of siRNAs (a 1:1:1 mixture of three siRNAs targeting the same gene) using Invitrogen RNAiMax transfection reagent. After an additional 24 h, cells were split 1:2 onto pre-coated 12-well plates and 4 h later were transfected with 500 ng ER_{GFP-NS} using TransIT-293 Transfection Reagent (Mirus). Media was changed after 24 h, and then cells were incubated for an additional ~20 h before cell extracts were made with NP40 lysis buffer (0.5% NP40, 50 mM Tris-HCl, pH 7.4, 2 mM MgCl₂, 150 mM NaCl, and 1 mM EDTA) plus 1,000x protease inhibitor and 0.5 mM TCEP (tris(2-carboxyethyl)phosphine). Cells were washed with cold 1x PBS, then 150 µl NP40 lysis buffer was added directly to wells and rocked for 30 min at 4°C. After lysis, lysate was transferred to a microcentrifuge tube and centrifuged at top speed for 5 min. The supernatant (120 µl) was collected in a clean microcentrifuge tube, and 40 µl of 4x Laemmli sample buffer (plus β-mercaptoethanol) was added to each tube. Samples were heated at 90°C for 15 min and loaded on an SDS-PAGE gel for immunoblotting analysis. siRNAs targeting UFM1, SAYSD1, and various TRAPP genes were reported previously (Wang et al., 2023). Other siRNAs are listed below in Table 1.

Immunostaining and immunoblotting

To prepare cells for immunofluorescence (IF) staining and imaging, cells grown and transfected in µ-Slide 8-well high-glass

Table 1. A list of siRNAs used in the study

siRNA	Manufacturer	Cat#
NEMF	Thermo Fisher Scientific	S17483
NEMF	Thermo Fisher Scientific	S17485
NEMF	Thermo Fisher Scientific	S17484
RNF160 [LTN1]	Thermo Fisher Scientific	S25004
RNF160 [LTN1]	Thermo Fisher Scientific	S25002
RNF160 [LTN1]	Thermo Fisher Scientific	S25003
KDELR2	Dharmacon	L-012315-00-0005
UFM1	Thermo Fisher Scientific	S28345
Silence Cy3-labeled negative control siRNA #1	Thermo Fisher Scientific	AM4621
ON-TARGET ^{plus} control pool nontargeting pool	Dharmacon	D-001810-10-05
Silencer select negative control siRNA #2	Thermo Fisher Scientific	4390847

bottom imaging chambers (Catalog# 80807; Ibidi) were washed once with ice-cold PBS, fixed with 4% paraformaldehyde in PBS for 15–20 min at room temperature, and then washed three times with PBS. Cells were treated with PBS containing 0.1% NP40 and 5% FBS for 15 min at room temperature and then stained for 1 h at room temperature with the primary antibodies listed below in Table 2. Stained cells were washed three times with PBS and then stained with the corresponding secondary antibody conjugated to a fluorescent dye (Alexa⁴⁸⁸, Alexa⁵⁶⁶, or Alexa⁶⁴⁰). In some experiments, cells were also stained with Hoechst 33342 to reveal the nuclei. Stained cells were washed three times with PBS.

For immunoblotting analyses, cell lysates or immunoprecipitated materials were fractionated on 4–12% NuPAGE gels following the manufacture's instruction (Thermo Fisher Scientific). Proteins were transferred to nitrocellulose membranes (BioRad) using a semidry protein transfer apparatus (Trans-Blot Turbo; BioRad). Membranes were treated with PBS containing 5% nonfat milk to block nonspecific binding and then incubated with primary antibodies in PBS containing 5% BSA as indicated below. After washing with PBS three times, we incubated the membranes for 1 h with HRP-conjugated or fluorescent secondary antibodies (Invitrogen). Chemiluminescent or fluorescent signals were detected by the BioRad ChemDoc scanning system.

Microscope image acquisition and processing

Fluorescence confocal microscopy images were collected on a Nikon CSU-W1 SoRa spinning disc super-resolution confocal microscope equipped with a 60× TIRF objective lens (NA = 1.6)

Table 2. A list of antibodies used in immunofluorescence (IF) staining and immunoblotting (IB)

Associated figures	Assay type	Protein name	Manufacturer	Catalogue no.	Dilution
Fig. 1 D	IF	Nucleolin	Bethyl	A300-711A	1:1,000
Fig. 1 J	IB	UBE1	Abcam	10R-P104A	1:1,000
Fig. 1 J	IB	Sec61 β	A gift from Tom Rapoport lab (Harvard Medical School, Boston, MA, USA)		1:5,000
Fig. 1, J and I; Fig. 2, C and D; Fig. 4, D and H	IB	GFP	Santa Cruz	SC-9996	1:500
Fig. 1 I; Fig. 2 C; Fig. 4, D and H	IB	UFM1	Abcam	Ab109305	1:1,000
Fig. 1 I; Fig. 3 D	IB	VCP/p97	Fitzgerald	10R-P104A	1:2,000
Fig. 2, C and D	IB	SAYSD1	Abcam	Ab189918	1:1,000
Fig. 2 D	IB	RPL26	Bethyl	A300-686A	1:1,000
Fig. 3 D	IB	Hrd1	Generated in-house Zhang et al. (2015)		1:1,000
Fig. 4 A	IF	GFP	Thermo Fisher Scientific	A11122	1:200
Fig. 4 C	IB	NEMF/ SDCCAG1	Proteintech	11840-1-AP	1:1,000
Fig. 4 C	IB	LTN1/RNF160	Proteintech	28452-1-AP	1:2,000
Fig. 4, C and H	IB	HSP90	Santa Cruz	SC-13119	1:1,000

and four laser lines (405, 488, 561, and 638 nm). Image collection was done using Nikon Element. Cells fixed and stained were imaged under an immersion oil. For live-cell imaging, cells were maintained in a temperature-controlled chamber incubator equipped with a CO₂ control. Temperature was set as 37°C. Acquired images were processed and analyzed by Fiji (ImageJ). Presented figures were made using Adobe Photoshop v 26.41 and Adobe Illustrator v 29.31.

Statistical analysis

Immunoblot data were analyzed with BioRad Image Lab. Imaging data were analyzed with ImageJ, Imaris, or Nikon Elements Analysis (live cell only). All statistical analyses were completed with GraphPad Prism 10 and are displayed as mean $\pm$ SEM.

Online supplemental material

[Fig. S1](#) shows that TAQC substrates can be degraded by either lysosomes or proteasome. [Fig. S2](#) shows the accumulation of ER_{GFP}_NS in the ER in SAYSD1 and TRAPPC8 knockdown cells. [Fig. S3](#) shows the CRISPR screen result for the UFMylation pathway genes and components of the TRAPP complex. Table S1 shows a list of statistically significant CRISPR screen hits.

Data availability

The data presented in this study are available in the published article and its online supplemental material.

Acknowledgments

We thank the Advanced Light Microscope Core at the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) for assistance with imaging, the NHLBI flow cytometry core for cell sorting, and the NHLBI Genomic Core for high-throughput

sequence. We also thank R. Kopito (Stanford University, Stanford, CA, USA) for the ssGFP plasmid.

The research is supported by an intramural research program of NIDDK and by the National Institutes of Health grant R35GM145249 (L. Huang).

Author contributions: A. Ennis: conceptualization, data curation, formal analysis, investigation, methodology, project administration, validation, visualization, and writing—original draft. L. Wang: conceptualization, data curation, formal analysis, investigation, methodology, and writing—review and editing. Y. Xu: formal analysis, methodology, and resources. L. Saidi: investigation and validation. X. Wang: data curation. C. Yu: investigation and visualization. S. Yun: formal analysis and software. L. Huang: funding acquisition and resources. Y. Ye: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, supervision, validation, visualization, and writing—original draft, review, and editing.

Disclosures: The authors declare no competing interests exist.

Submitted: 27 August 2024

Revised: 19 January 2025

Accepted: 20 March 2025

References

- Arakawa, S., K. Yunoki, T. Izawa, Y. Tamura, S. Nishikawa, and T. Endo. 2016. Quality control of nonstop membrane proteins at the ER membrane and in the cytosol. *Sci. Rep.* 6:30795.
- Barrowman, J., D. Bhandari, K. Reinisch, and S. Ferro-Novick. 2010. TRAPP complexes in membrane traffic: Convergence through a common Rab. *Nat. Rev. Mol. Cell Biol.* 11:759–763. <https://doi.org/10.1038/nrm2999>
- Bhattacharya, A., and L. Qi. 2019. ER-associated degradation in health and disease - from substrate to organism. *J. Cell Sci.* 132:jcs.232850. <https://doi.org/10.1242/jcs.232850>

- Christianson, J.C., and Y. Ye. 2014. Cleaning up in the endoplasmic reticulum: Ubiquitin in charge. *Nat. Struct. Mol. Biol.* 21:325–335. <https://doi.org/10.1038/nsmb.2793>
- Crowder, J.J., M. Geigges, R.T. Gibson, E.S. Fufts, B.W. Buchanan, N. Sachs, A. Schink, S.G. Kreft, and E.M. Rubenstein. 2015. Rkr1/Ltn1 ubiquitin ligase-mediated degradation of translationally stalled endoplasmic reticulum proteins. *J. Biol. Chem.* 290:18454–18466. <https://doi.org/10.1074/jbc.M115.663559>
- Dimitrova, L.N., K. Kuroha, T. Tatematsu, and T. Inada. 2009. Nascent peptide-dependent translation arrest leads to Not4p-mediated protein degradation by the proteasome. *J. Biol. Chem.* 284:10343–10352. <https://doi.org/10.1074/jbc.M808840200>
- Filbeck, S., F. Cerullo, S. Pfeffer, and C.A.P. Joazeiro. 2022. Ribosome-associated quality-control mechanisms from bacteria to humans. *Mol. Cell.* 82:1451–1466. <https://doi.org/10.1016/j.molcel.2022.03.038>
- Howard, C.J., and A. Frost. 2021. Ribosome-associated quality control and CAT tailing. *Crit. Rev. Biochem. Mol. Biol.* 56:603–620. <https://doi.org/10.1080/10409238.2021.1938507>
- Inada, T. 2020. Quality controls induced by aberrant translation. *Nucleic Acids Res.* 48:1084–1096. <https://doi.org/10.1093/nar/gkz1201>
- Joazeiro, C.A.P. 2019. Mechanisms and functions of ribosome-associated protein quality control. *Nat. Rev. Mol. Cell Biol.* 20:368–383. <https://doi.org/10.1038/s41580-019-0118-2>
- Juszkiewicz, S., and R.S. Hegde. 2017. Initiation of quality control during Poly(A) translation requires site-specific ribosome ubiquitination. *Mol. Cell.* 65:743–750.e4. <https://doi.org/10.1016/j.molcel.2016.11.039>
- Kostova, K.K., K.L. Hickey, B.A. Osuna, J.A. Hussmann, A. Frost, D.E. Weinberg, and J.S. Weissman. 2017. CAT-tailing as a fail-safe mechanism for efficient degradation of stalled nascent polypeptides. *Science*. 357:414–417. <https://doi.org/10.1126/science.aam7787>
- Lv, L., J. Mo, Y. Qing, S. Wang, L. Chen, A. Mei, R. Xu, H. Huang, J. Tan, Y. Li, and J. Liu. 2024. NEMF-mediated Listerin-independent mitochondrial translational surveillance by E3 ligase Pirh2 and mitochondrial protease ClpXP. *Cell Rep.* 43:113860. <https://doi.org/10.1016/j.celrep.2024.113860>
- Lyumkis, D., D. Oliveira dos Passos, E.B. Tahara, K. Webb, E.J. Bennett, S. Vinterbo, C.S. Potter, B. Carragher, and C.A. Joazeiro. 2014. Structural basis for translational surveillance by the large ribosomal subunit-associated protein quality control complex. *Proc. Natl. Acad. Sci. USA*. 111:15981–15986. <https://doi.org/10.1073/pnas.1413882111>
- Martin, M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet J.* 17:10–12. <https://doi.org/10.14806/ej.17.1.200>
- Martin, R.M., G. Ter-Avetisyan, H.D. Hecce, A.K. Ludwig, G. Lättig-Tünnemann, and M.C. Cardoso. 2015. Principles of protein targeting to the nucleolus. *Nucleus*. 6:314–325. <https://doi.org/10.1080/19491034.2015.1079680>
- Mizuno, M., S. Ebine, O. Shounai, S. Nakajima, S. Tomomatsu, K. Ikeuchi, Y. Matsuo, and T. Inada. 2021. The nascent polypeptide in the 60S subunit determines the Rqc2-dependency of ribosomal quality control. *Nucleic Acids Res.* 49:2102–2113. <https://doi.org/10.1093/nar/gkab005>
- Ruggiano, A., O. Foresti, and P. Carvalho. 2014. Quality control: ER-associated degradation: Protein quality control and beyond. *J. Cell Biol.* 204:869–879. <https://doi.org/10.1083/jcb.201312042>
- Sanjana, N.E., O. Shalem, and F. Zhang. 2014. Improved vectors and genome-wide libraries for CRISPR screening. *Nat. Methods*. 11:783–784. <https://doi.org/10.1038/nmeth.3047>
- Scavone, F., S.C. Gumbin, P.A. Da Rosa, and R.R. Kopito. 2023. RPL26/uL24 UFMylation is essential for ribosome-associated quality control at the endoplasmic reticulum. *Proc. Natl. Acad. Sci. USA*. 120:e2220340120. <https://doi.org/10.1073/pnas.2220340120>
- Shamu, C.E., C.M. Story, T.A. Rapoport, and H.L. Ploegh. 1999. The pathway of US11-dependent degradation of MHC class I heavy chains involves a ubiquitin-conjugated intermediate. *J. Cell Biol.* 147:45–58. <https://doi.org/10.1083/jcb.147.1.45>
- Shao, S., A. Brown, B. Santhanam, and R.S. Hegde. 2015. Structure and assembly pathway of the ribosome quality control complex. *Mol. Cell.* 57:433–444. <https://doi.org/10.1016/j.molcel.2014.12.015>
- Shen, P.S., J. Park, Y. Qin, X. Li, K. Parsawar, M.H. Larson, J. Cox, Y. Cheng, A.M. Lambowitz, J.S. Weissman, et al. 2015. Protein synthesis. Rqc2p and 60S ribosomal subunits mediate mRNA-independent elongation of nascent chains. *Science*. 347:75–78. <https://doi.org/10.1126/science.1259724>
- Sitron, C.S., and O. Brandman. 2019. CAT tails drive degradation of stalled polypeptides on and off the ribosome. *Nat. Struct. Mol. Biol.* 26:450–459. <https://doi.org/10.1038/s41594-019-0230-1>
- Tesina, P., S. Ebine, R. Buschauer, M. Thoms, Y. Matsuo, T. Inada, and R. Beckmann. 2023. Molecular basis of eIF5A-dependent CAT tailing in eukaryotic ribosome-associated quality control. *Mol. Cell.* 83:607–621.e4. <https://doi.org/10.1016/j.molcel.2023.01.020>
- Thrun, A., A. Garzia, Y. Kigoshi-Tansho, P.R. Patil, C.S. Umbaugh, T. Dalling, J. Liu, S. Kreger, A. Patrizi, G.A. Cox, et al. 2021. Convergence of mammalian RQC and C-end rule proteolytic pathways via alanine tailing. *Mol. Cell.* 81:2112–2122.e7. <https://doi.org/10.1016/j.molcel.2021.03.004>
- Vembar, S.S., and J.L. Brodsky. 2008. One step at a time: Endoplasmic reticulum-associated degradation. *Nat. Rev. Mol. Cell Biol.* 9:944–957. <https://doi.org/10.1038/nrm2546>
- Walczak, C.P., D.E. Leto, L. Zhang, C. Riepe, R.Y. Muller, P.A. DaRosa, N.T. Ingolia, J.E. Elias, and R.R. Kopito. 2019. Ribosomal protein RPL26 is the principal target of UFMylation. *Proc. Natl. Acad. Sci. USA*. 116:1299–1308. <https://doi.org/10.1073/pnas.1816202116>
- Wang, L., Y. Xu, H. Rogers, L. Saidi, C.T. Noguchi, H. Li, J.W. Yewdell, N.R. Guydosh, and Y. Ye. 2020. UFMylation of RPL26 links translocation-associated quality control to endoplasmic reticulum protein homeostasis. *Cell Res.* 30:5–20. <https://doi.org/10.1038/s41422-019-0236-6>
- Wang, L., Y. Xu, S. Yun, Q. Yuan, P. Satpute-Krishnan, and Y. Ye. 2023. SAYSD1 senses UFMylated ribosome to safeguard co-translational protein translocation at the endoplasmic reticulum. *Cell Rep.* 42:112028. <https://doi.org/10.1016/j.celrep.2023.112028>
- Wang, L., and Y. Ye. 2021. Clearing traffic jams during protein translocation across membranes. *Front. Cell Dev. Biol.* 8:610689. <https://doi.org/10.3389/fcell.2020.610689>
- Ye, Y., H.H. Meyer, and T.A. Rapoport. 2001. The AAA ATPase Cdc48/p97 and its partners transport proteins from the ER into the cytosol. *Nature*. 414:652–656. <https://doi.org/10.1038/414652a>
- Yip, M.C.J., and S. Shao. 2021. Detecting and rescuing stalled ribosomes. *Trends Biochem. Sci.* 46:731–743. <https://doi.org/10.1016/j.tibs.2021.03.008>
- Zhang, T., Y. Xu, Y. Liu, and Y. Ye. 2015. gp78 functions downstream of Hrd1 to promote degradation of misfolded proteins of the endoplasmic reticulum. *Mol. Biol. Cell.* 26:4438–4450. <https://doi.org/10.1091/mbc.E15-06-0354>

Supplemental material

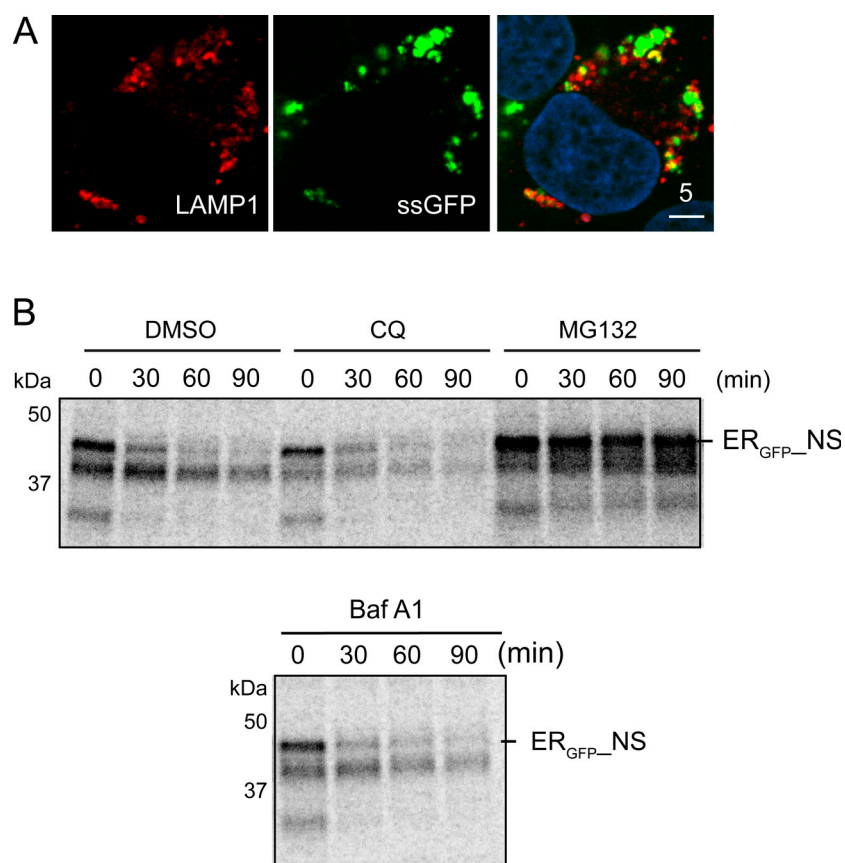


Figure S1. **TAQC substrates can be degraded by lysosomes and the proteasome.** (A) ssGFP_K20-transfected cells were treated with Baf A1 (200 nM 4 h) and stained with LAMP1 antibodies (red). Note the partial localization of ssGFP_K20 with LAMP1. Scale bar, 5 μ m. (B) ERGFP_NS-expressing HEK293T cells treated with the indicated compounds were pulse labeled with S35-Met and chase in a medium containing unlabeled S35-Met. Samples taken at the indicated time points were subject to immunoprecipitation and autoradiography. CQ, chloroquine. Source data are available for this figure: SourceData FS1.

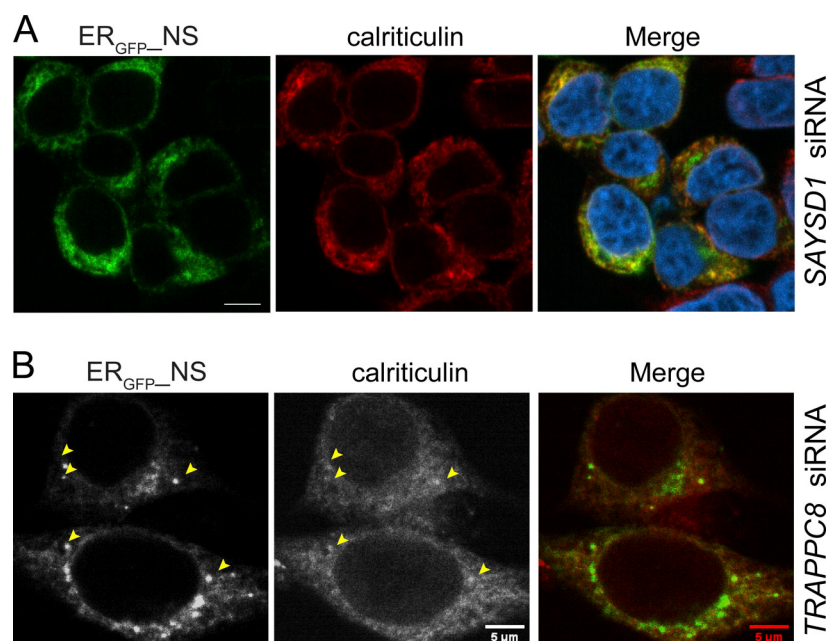


Figure S2. **ERGFP_NS accumulates in the ER in SAYSD1 and TRAPPC8 knockdown cells.** (A) HEK293T cells transfected with ERGFP_NS and SAYSD1 siRNA were stained with calreticulin antibodies (red) and Hoechst (blue). Scale bar, 5 μ m. (B) HEK293T cells transfected with ERGFP_NS and TRAPPC8 siRNA were stained with calreticulin antibodies. Arrowheads indicate a few examples of ERGFP_NS and calreticulin co-localization. Scale bar, 5 μ m.

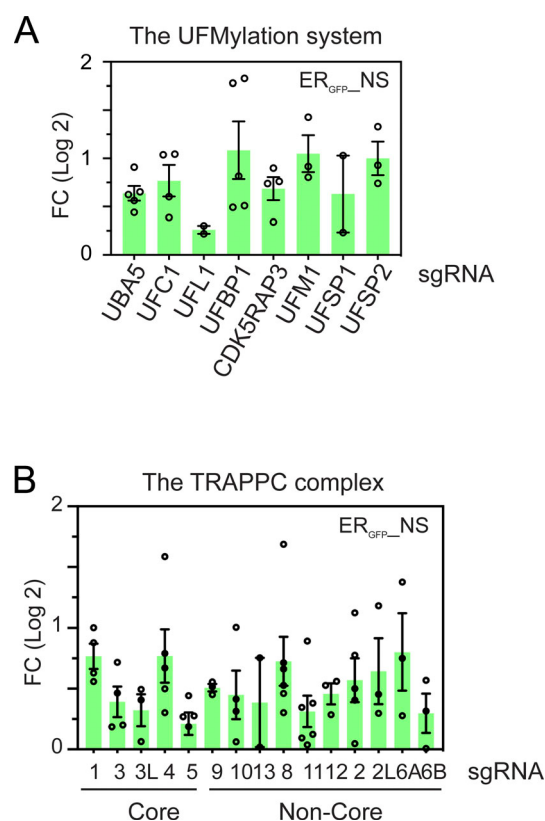


Figure S3. **Genetic screen reveals the UFMylation system and the TRAPPC complex as regulators of ERGFP_NS degradation.** (A and B) sgRNAs targeting genes of the UFMylation system (A) or the TRAPPC complex (B) are positively enriched in GFP-high population, suggesting they are positive regulators for ERGFP_NS degradation. FC, fold change.

Provided online is Table S1. Table S1 shows a list of sgRNAs enriched in GFP high cells in ERGFP_NS screen.