SI Appendix:

RPL26/uL24 UFMylation is essential for ribosome-associated quality control at the endoplasmic reticulum

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Figure S1. Related to Figure 1. Characterization of ER-targeted ribosome stalling reporters.

A: The UFMylation system shown in relation to the ER membrane bilayer. UFM1 conjugation machinery (E1: UBA5, E2: UFC1, E3: UFL1, CDK5RAP3, DDRGK1) and deconjugation machinery (UFSP1, UFSP2, ODR4) are shown in their associated complexes. The target of UFMylation, RPL26, is shown in the context of the 60S ribosomal subunit.

B: PNGase F treatment on SS^{gVgV} reporter confirms correct ER targeting of SS^{gVgV} by increase in AP and RT mobility. Glycosylated (+2g) and nonglycosylated RT and AP species are indicated; data shown are representative of three independent experiments.

C: ER-stalled ribosomes are recognized and split by ZNF598 and ASCC3, respectively. HEK293 cells were transfected with scrambled (SCR), ZNF598, or ASCC3 small interfering RNAs (siRNA) and stalling reporter constructs $Cyto^{VV}$, SS^{VV}, SS^{VgV}. Immunoblot analysis was performed on WCLs using FLAG antibody to monitor changes in steady-state levels of frameshift (FS), RT, and AP species. Asterisks indicate nonspecific immunoreactive bands. Data shown are representative of three independent experiments.

D: Cell fractionation analysis of subcellular $Cyto^{VV}$-AP distribution. HEK293 cells were transfected with $Cyto^{VV}$ and subjected to cell fractionation. Reporter products were analyzed by immunoblot of WCL, Cyto, and ER cell fractions with FLAG antibody. GAPDH: cytosol marker; SEC61β: ER marker; data shown are representative of three independent experiments.

E: Titration of digitonin to determine the optimal concentration to solubilize the luminal contents of the ER (Sup) without extracting ER membrane proteins (Pellet). PDI: ER lumen marker; SEC61β and HRD1: ER membrane markers. Red box indicates the optimal digitonin concentration to separate ER luminal proteins (“ER-Lum”) from ER membrane proteins (“ER-Mem”) for the experiment in Figure 1G; data shown are representative of two independent experiments.

F: Ribosome association of ER-APs assessed by sedimentation. HEK293 cells were transfected with SS^{gVgV} and subjected to sucrose cushion sedimentation. Reporter products were analyzed by immunoblot of WCL, ribosome-free (Sup), and ribosome-associated (Pellet) fractions with FLAG antibody. RPL17: loading control.

Figure S2. Related to Figure 2. Validation data for ER-AP degradation via the proteasome.

A: Prolonged BafA treatment does not result in ER-AP stabilization. HEK293 cells were transfected with the ER targeted stalling reporters, SS^{VgV} (“Original” reporter, see figure S2F) and SS^{VV}, incubated with DMSO, 1 µM BTZ for 4 hr, or 100 nM BafA for 4 hr or 16 hr. WCLs were analyzed by immunoblot with FLAG antibody to detect AP and AP+g species, and LC3 antibody to assess the effect of BafA treatment on LC3-II

accumulation. GAPDH: loading control; data shown are representative of two independent experiments.
B: BTZ treatment promotes accumulation of a nonglycosylated SS^{VgV} ER-AP species. HEK293 cells were transfected with the indicated reporter and treated with DMSO or 1 µM BTZ for 4 hr. Reporter products were analyzed by immunoblot of WCLs with FLAG antibody. Labels indicate mobilities of glycosylated (+g) and non-glycosylated RT and APs; data shown are representative of three independent experiments.
C: Cell fractionation analysis of subcellular $Cyto^{VV}$-AP distribution following BTZ treatment. U2OS cells were transfected with the indicated reporter and treated with DMSO or 1 µM BTZ for 4 hr prior to cell fractionation. Reporter products were analyzed by immunoblot of WCL, Cyto, and ER cell fractions with FLAG antibody. GAPDH: cytosol marker; SEC61β: ER marker.
D: Validation of BTZ and NMS-873 efficacy. HEK293 cells transfected with the indicated reporters were treated for 4 hr with 1 µM BTZ or 5 µM NMS-873. Reporter products were analyzed by immunoblot with FLAG antibody. Immunoblotting with anti-CD147 antibody was performed to assess the effect of BTZ and NMS-873 on steady state levels of CD147, an endogenous proteasome substrate. Mat: Mature glycosylated CD147; CG: Core Glycosylated CD147; Degly: Deglycosylated CD147; data shown are representative of two independent experiments.
E: A GFP-based stalling reporter is degraded by the proteasome. *Upper panel,* Schematic of GFP-based stalling reporter, SS^{GFP}. Reporter contains SS^{PPL}, signal sequence from bovine preprolactin; EGFP, enhanced green fluorescent protein; FLAG, FLAG epitope tag. Composition of the reporter is shown below. The predicted MW for arrest peptide (AP, black line) is 50 kDa. *Lower panel,* HEK293 cells expressing SS^{GFP} were treated either with DMSO, 1 µM bortezomib (BTZ), or 100 nM Bafilomycin A1 (BafA) for 4 hr. WCLs were analyzed by immunoblot with FLAG antibody to detect AP species, and LC3 antibody to assess the effect of BafA treatment on LC3-II accumulation. GAPDH: loading control. *Right Panel,* Topological organization of GFP arrest peptide when stalled at the translocon. When the ribosome translating SS^{GFP} reaches the stall sequence, the entire GFP protein will be translocated into the ER lumen and folded.
F: Schematic of the two reporter variants illustrating the frameshift (FS) species generated by our stalling reporters as described in the materials and methods section. Original: frameshift product generated by this reporter is ~25kD. FS-corrected: frameshift product generated by this reporter is 60kD or 65kD. S Tag+1 and S Tag+2 are generated by out of frame translation downstream of the stalling sequence (K20). The Original reporter was used in Figures 2E, S2A. The FS-corrected reporter is used in all other experiments.

Figure S3. Related to Figure 3. Extended data for the requirement of RQC machinery to degrade ER-APs.

A: Control for Fig 3A. Effect of scrambled (SCR), LTN1, or HRD1 siRNA on endogenous protein levels, assayed by immunoblot for endogenous LTN1 or HRD1 with anti-LTN1 or anti-HRD1 antibody, respectively.

B-D: Estimation of CAT tail length for ER-APs.

B: Representative 12% SDS-PAGE used to determine the molecular weight (MW) of ER-AP CAT tails. As in Fig 3E, HEK293 *NEMF*^{KO} cells were rescued with WT NEMF or NEMF-DR and transfected with the SS^{VV} stalling reporter. WCLs were analyzed by immunoblot with anti-FLAG antibody. Unmodified APs are indicated by the label "AP" and by black arrowheads; CATylated APs are indicated by "AP^{CAT}". Blue arrows indicate migration distance (inches) from the top of the gel. GAPDH and tubulin: loading controls.

C: Semi-logarithmic plot of MW of size markers versus migration distance (inches) from Fig S3B. Data shown are representative of four experiments. The equation of the best fit line (y = -0.34x + 2.03) is used to calculate the MW of the AP species in this experiment.

D: Summary table of four independent measurements for SS^{VV} CAT tail length. MW of AP or AP^{CAT} was estimated by linear regression analysis of semi-logarithmic plots of MW of size markers versus migration distance of AP or AP^{CAT} in 12% SDS-PAGE.

Figure S4. Related to Figure 4. Extended data on the requirement of UFMylation for ER-AP degradation.

A: ER-targeted stalls specifically promote RPL26 UFMylation. *Upper panel,* HEK293 cells were transfected with 2 µgs of empty vector (EV) or indicated nonstall (K0) or stall (K20) reporter plasmids. 20% of WCLs from samples transfected with K0 reporters were loaded compared to WCLs transfected with K20 stall reporters. Where indicated cells were treated with 200 nM ANS (+ANS) for 15 minutes prior to cell lysis. WCLs were analyzed by immunoblot with anti-FLAG antibody. *Lower panel,* WCLs were sedimented through a sucrose cushion and the pellets were analyzed by immunoblot with anti-UFM1 antibody. RPL17: loading control.

B: Cytosolic stalling reporter does not induce UFMylation. *Upper panel,* HEK293 cells were transfected with 4 or 5 µgs of empty vector (EV) or Cyto^{VV}-K20. Where indicated cells were treated with 200 nM ANS (+ANS) for 15 minutes prior to cell lysis. GAPDH: loading control. *Lower panel,* WCLs were sedimented through a sucrose cushion and the pellets were analyzed by immunoblot with anti-UFM1 antibody. RPL17: loading control.

C: ER stalling reporters with diverse sequences and topologies induce RPL26 UFMylation. *Left panels,* Schematic of the stalling reporters used in this experiment. TFR^{TMD}, transmembrane domain from transferrin receptor; SS^{PPL}, signal sequence from bovine preprolactin; PPL^{FL}, prolactin; FLAG, FLAG epitope tag; glyc, position of an N-glycosylation site or "sequon"; "Y", N-glycan; VHP, villin headpiece domain; K20,

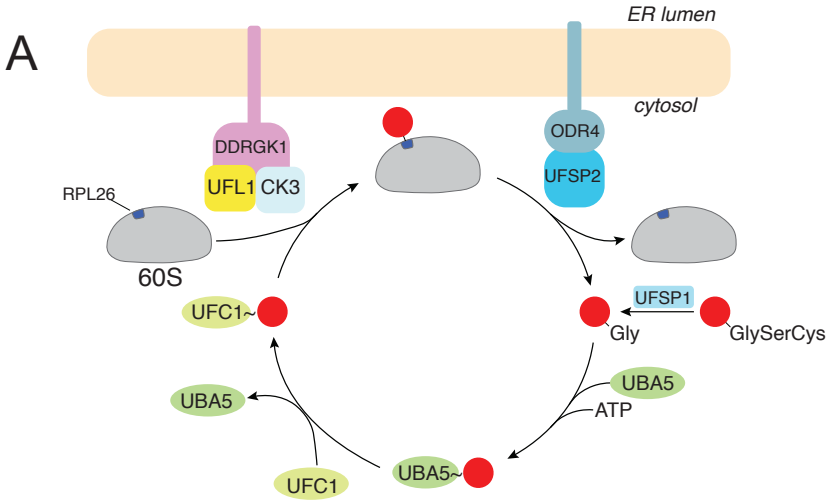
indication that the reporter contains a polylysine stalling sequence of 20 lysine residues; GFP, superfolder green fluorescent protein; V5, epitope tag; HA, epitope tag. Composition of each reporter shown below, with predicted MW for arrest peptide (AP, black line) or readthrough (RT, black line + dashed black line) species produced by each stalling reporter. *Right panel,* HEK293 cells were transfected with 2µgs of empty vector (EV) or the indicated stall (K20) reporters. Where indicated cells were treated with 200 nM ANS (+ANS) for 15 minutes prior to cell lysis. WCLs were analyzed by immunoblot with anti-FLAG antibody. Tubulin: loading control. *Lower panel,* WCLs were sedimented through a sucrose cushion and the pellets were analyzed by immunoblot with anti-UFM1 antibody. RPL17: loading control.

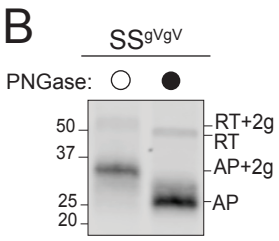
D: *UFM1^{KO}* stabilizes ER but not cytosolic-APs in U2OS cells, as in HEK293 cells (figure 4D). U2OS WT and clonal *UFM1^{KO}* cell lines were transfected with the indicated reporters. Reporter products were analyzed by immunoblot with FLAG antibody. Knockout was confirmed by immunoblot with anti-UFM1 antibody. GAPDH: loading control.

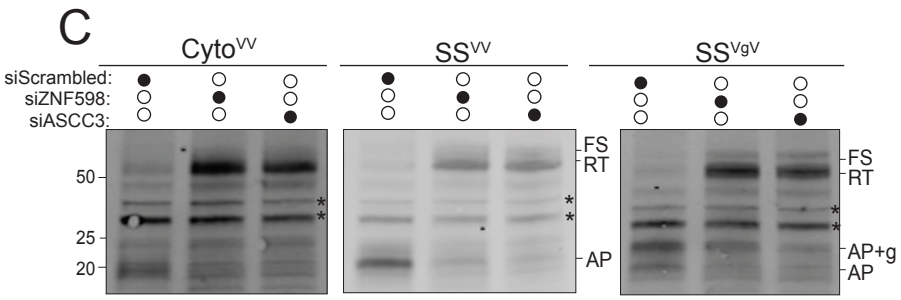
E: ER-AP degradation requires UFM1 conjugation machinery. HEK293 WT, *UFM1^{KO}*, *UFC1^{KO}*, and *UFL1^{KO}* cell lines were transfected with the indicated reporters. Reporter products were analyzed by immunoblot with FLAG antibody. Knockouts were confirmed by blotting with antibodies against endogenous UFC1 or UFL1 proteins with anti-UFC1 or anti-UFL1 antibodies, respectively. GAPDH: loading control; data shown are representative of two independent experiments.

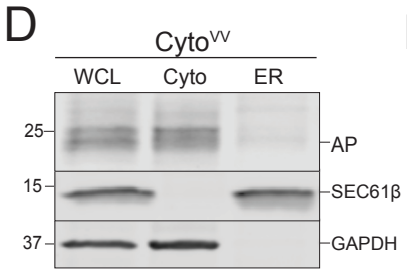
F: Validation of U2OS UBA5^{DD} cells. UBA5 and RPL26-UFM1 are undetectable 24 hr after washout. U2OS UBA5^{DD} cells were cultured in complete DMEM with TMP ("-Washout") or washed to remove TMP from the media ("+Washout") at the indicated times. *Upper panel,* WCLs were sedimented through a 1M sucrose cushion and the pellets were analyzed by immunoblot with anti-UFM1 antibody. RPL17: loading control. *Middle panel,* WCLs from this experiment were analyzed by immunoblot with anti-UBA5 antibody to assess levels of remaining DHFR-UBA5. *Lower panel,* WCLs were analyzed by immunoblot with anti-UFM1 to detect conjugates with UFC1 and DHFR-UBA5 which are lost after acute depletion of UBA5. Asterisks indicate nonspecific immunoreactive bands; data shown are representative of two independent experiments.

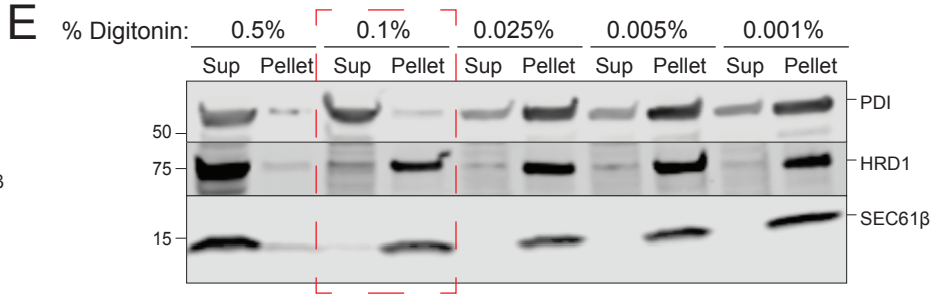
Supplemental Figure 1

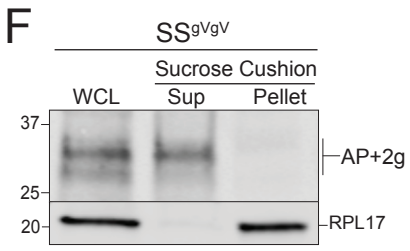




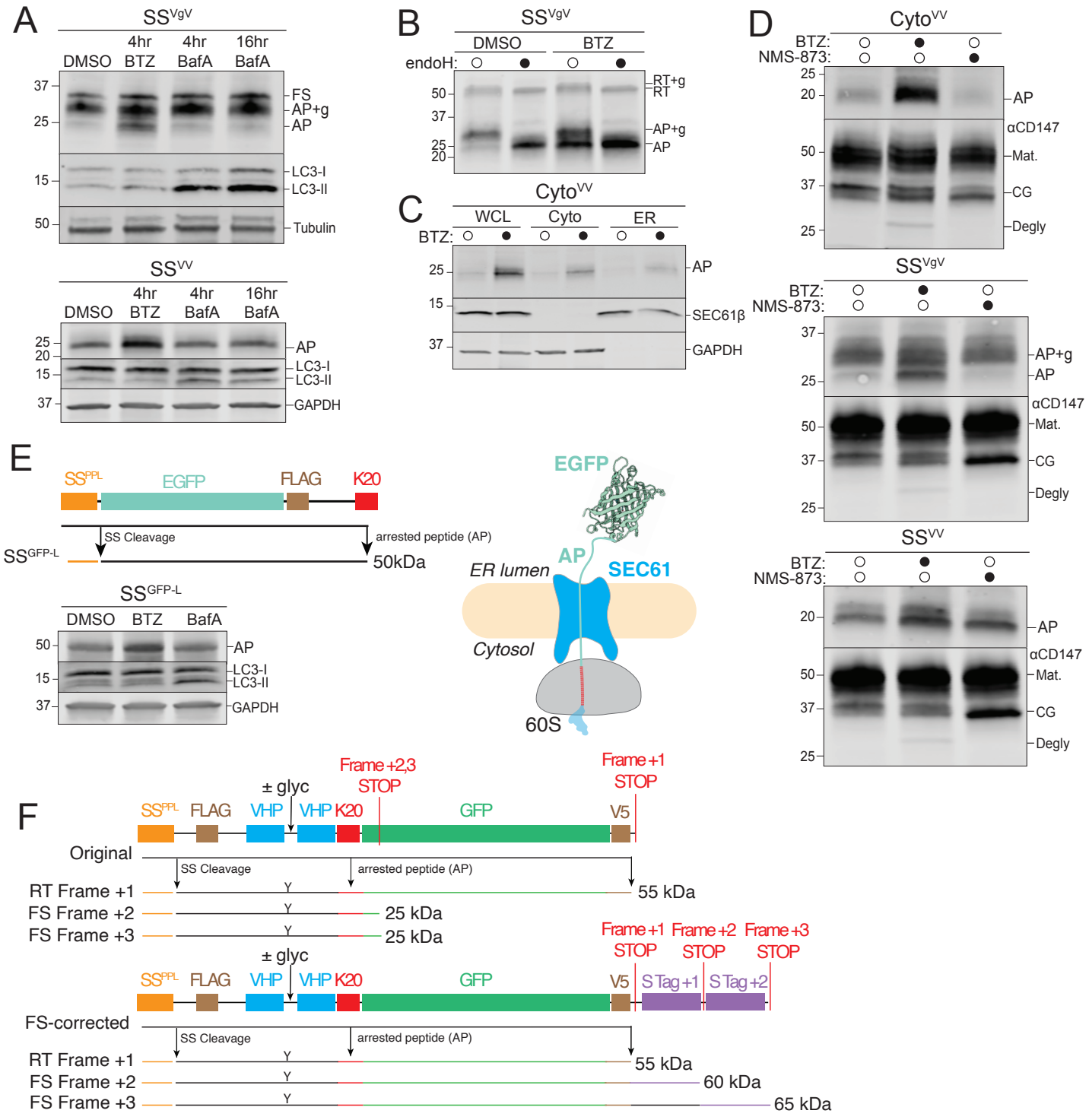






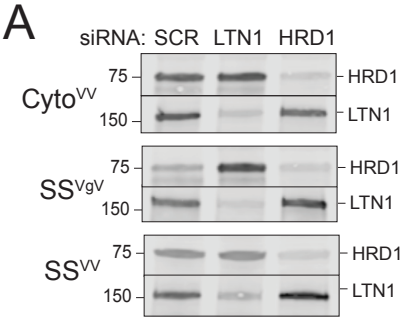


Supplemental Figure 2



Supplemental Figure 3

A



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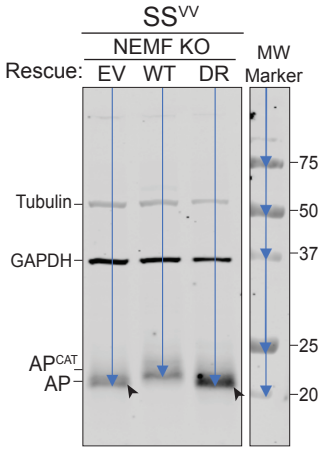

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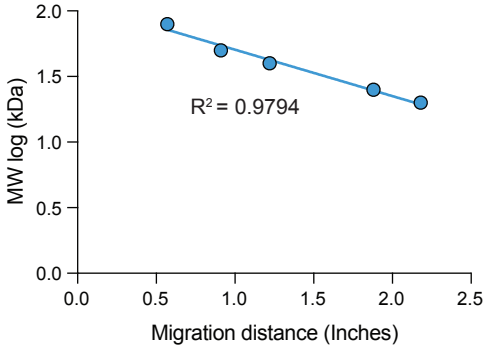
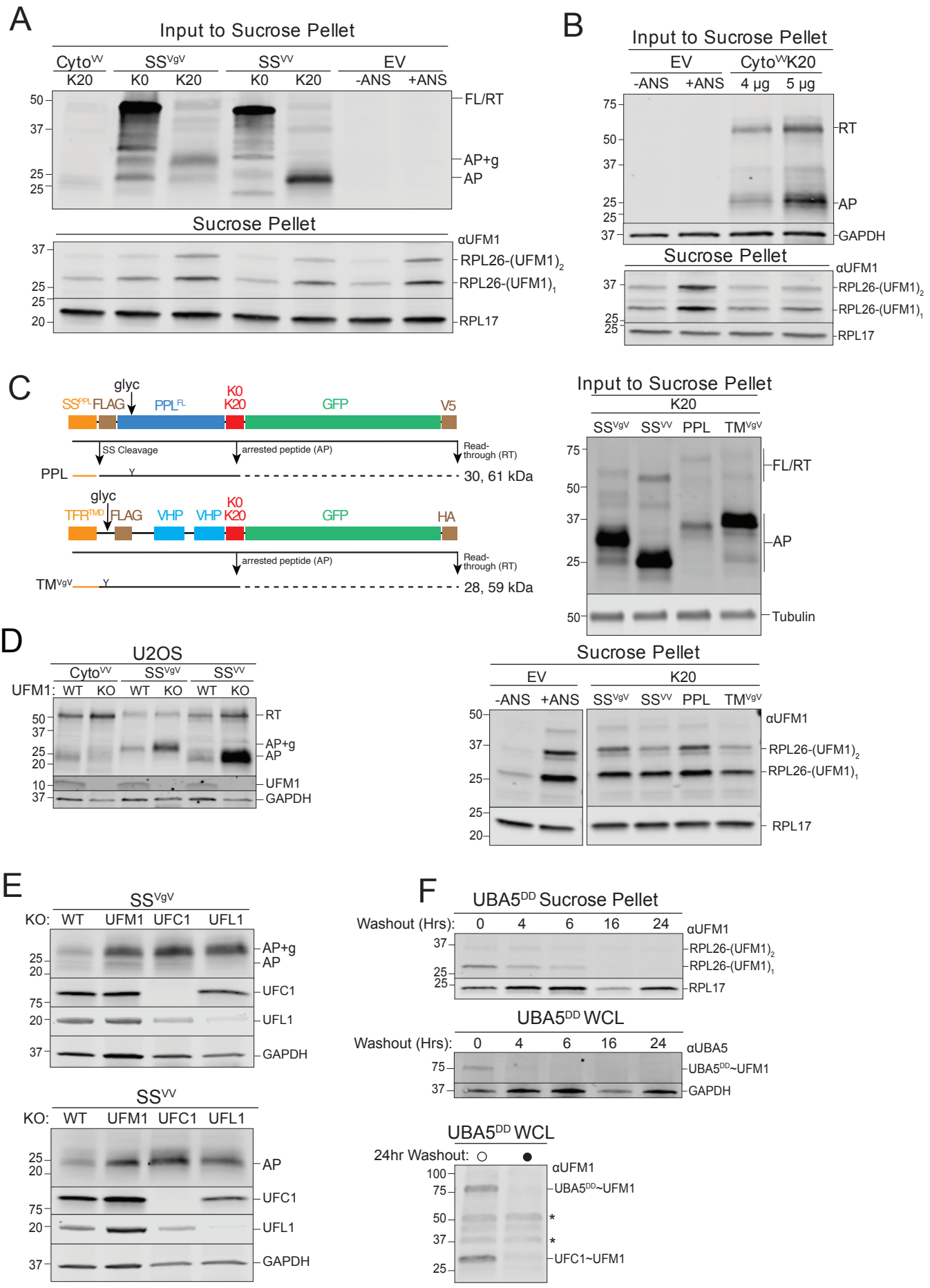


D

	Expt 1	Expt 2	Expt 3	Expt 4
AP^{CAT}(kDa)	22.2	21.7	21.4	23.4
AP(kDa)	20.9	20.5	20.2	22.1
CAT tail length (kDa)	1.3	1.2	1.2	1.3

Supplemental Figure 4



Reagent or Resource	Source	Identifier (CAT #)
Antibodies		
Mouse monoclonal anti-FLAG	Sigma-Aldrich	F1804
Mouse monoclonal anti-GAPDH	Cell Signaling	97166S
Rabbit polyclonal anti-GAPDH	Cell Signaling	2118S
Mouse monoclonal anti-PDI	Enzo Life Sciences	ADI-SPA-891
Rabbit polyclonal anti-SEC61β	Gift from Hegde Lab	N/A
Rabbit polyclonal anti-RPS10	Abclonal technologies	A6056
Rabbit polyclonal anti-ASCC3	Proteintech	17627-1-AP
Rabbit polyclonal anti-Tubulin	Abcam	ab15246
Rabbit polyclonal anti-HRD1	Proteintech	13473-1-AP
Mouse monoclonal anti-RPL17	Santa Cruz Biotechnology	sc-515904
Rabbit polyclonal anti-LC3B	Cell Signaling	2775S
Rabbit polyclonal anti-SEL1L	Abcepta)	N/A
Mouse monoclonal anti-CD147	Santa Cruz Biotechnology	sc-21746
Rabbit polyclonal anti-LTN1	Proteintech	28452-1-AP
Rabbit polyclonal anti-NEMF	Thermo Fisher Scientific	PA5-36308
Rabbit monoclonal anti-UFM1	Abcam	ab109305
Rabbit monoclonal anti-UFC1	Abcam	ab189252
Rabbit monoclonal anti-UFL1	Abcam	ab227506
Rabbit polyclonal anti-UBA5	Proteintech	12093-1-AP
Rabbit polyclonal anti-RPL26	Abcam	ab59567
Goat anti-Mouse IgG, IRDye 800CW	LI-COR Biosciences	926-32210
Goat anti-Mouse IgG, IRDye 680LT	LI-COR Biosciences	926-68020
Goat anti-Rabbit IgG, IRDye 800CW	LI-COR Biosciences	926-32211
Goat anti-Rabbit IgG, IRDye 680LT	LI-COR Biosciences	926-68021
Bacterial and Virus Strains		
E. coli: MAX efficiency DH5α competent cells	Thermo Fisher Scientific	18258012E
Chemicals, Peptides, and Recombinant Proteins		
cOmplete, EDTA-free Protease Inhibitor Cocktail	Sigma-Aldrich	11873580001
Doxycycline hyclate	Sigma-Aldrich	D9891
Lipofectamine 3000	Thermo Fisher Scientific	L3000015
Lipofectamine RNAiMAX	Life Technologies/Invitrogen	13778100
Emetine dihydrochloride hydrate	Sigma-Aldrich	E2375
NMS-873	Sigma-Aldrich	SML1128
Puromycin dihydrochloride	Thermo Fisher Scientific	A1113803
Bortezomib	Selleck Chemicals	S1013
Phenylmethylsulfonyl fluoride (PMSF)	Sigma-Aldrich	10837091001
Odessey Blocking Buffer	LI-COR Biosciences	927-60003
Endo H	New England Biolabs	P0702S
PNGase F	New England Biolabs	P0704S
N-ethylmaleimide	Sigma-Aldrich	E3876
Phusion Master Mix with HF Buffer	Thermo Fisher Scientific	F-531L
Dithiothreitol (DTT)	Fisher Scientific	ab000490-00010

TransIT LT1 transfection reagent	Mirus Bio LLC	MIR 2300
Anisomycin	Sigma-Aldrich	A9789
Bafilomycin A1	Sigma-Aldrich	B1793
Digitonin	EMD Millipore	300410
Sucrose	Sigma-Aldrich	S8501
TMP	Sigma-Aldrich	T7883
HyClone Dulbecco's Modified Eagle Medium (DMEM) with high glucose	Cytiva	SH30285.FS
Critical Commercial Assays		
PCR Mycoplasma Detection Kit	ABM inc.	G238
BCA Protein Assay Kit	Thermo Fisher Scientific	23225
660 nm Protein Assay Reagent	Thermo Fisher Scientific	22660
QIAprep Spin Miniprep Kit	Qiagen	27106X4
PureLink™ HiPure Plasmid Midiprep Kit	Thermo Fisher Scientific	K210004
Experimental Models: Cell Lines		
Human: HEK293 Cells	ATCC	CRL-1573
Human: HEK293T Cells	ATCC	CRL-3216
Human: U2OS Cells	ATCC	HTB-96
Human: HEK293 HRD1 KO Cells	(van der Goot et al., 2018)	N/A
Human: HEK293 OS9 KO Cells	(van der Goot et al., 2018)	N/A
Human: HEK293 SEL1L KO Cells	(van der Goot et al., 2018)	N/A
Human: HEK293 LTN1 KO Cells	This study	N/A
Human: HEK293 NEMF KO Cells	This study	N/A
Human: HEK293 UFM1 KO Cells	(Walczak et al., 2019)	N/A
Human: HEK293 UFC1 KO Cells	(Walczak et al., 2019)	N/A
Human: HEK293 UFL1 KO Cells	(Walczak et al., 2019)	N/A
Human: U2OS UBA5-DD Cells	This study	N/A
Human: U2OS UFM1 KO Cells	(Walczak et al., 2019)	N/A
Human: HEK293 FLAG-Cas9 Cells	Gift from Ting Lab	N/A
Human: HEKT Cells	Gift from Yi Lab	N/A
Human: HEKT RPL26ΔC	Gift from Yi Lab	N/A
Oligonucleotides		
Silencer™ Select Negative Control No. 1 siRNA	Thermo Fisher Scientific	4390843
siRNA against ZNF598	Thermo Fisher Scientific	4392420
siRNA against ASCC3	Thermo Fisher Scientific	s21603
siRNA against LTN1	Thermo Fisher Scientific	s25003
siRNA against HRD1	Thermo Fisher Scientific	4427037
siRNA against NEMF	Thermo Fisher Scientific	s17485
SS Bovine Preprolactin Fw: GGATCCACCatggacagcaaaggttcgtcgcagaaagggtcccgcctgctcctgctgctggtggtgtcaaatctactcttgtgccagggtgtggtctccacc TCAGGGTCTGGTAGCGGCGGCCGC	This study	N/A
SS Bovine Preprolactin Rev: GCGGCCGCCGCTACCAGACCCTGAggtggagaccacaccctggcacaagagtagatttgacaccaccagcagcaggagcaggcgggaccc tttctgcgacgaacctttgctgtccatGGTGGATCC	This study	N/A
K20 Stall Sequence Fw: CAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAGCTAAGCC	This study	N/A
K20 Stall Sequence Rev: GGCTTAGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTG	This study	N/A

Recombinant DNA		
sgRNA NEMF (sequence: GACCTCCGCGCCGTACTCG)	This study	N/A
sgRNA LTN1 (sequence: AATGCCGAACGACAGTGAA)	This study	N/A
sgRNA UBA5 (sequence: ACCTACTATTGCTACGGCAA)	(Walczak et al., 2019)	N/A
Plasmid: pcDNA3.1 (+)	Thermo Fisher Scientific	V79020
Plasmid: UBA5 pDONOR-STOP	(Walczak et al., 2019)	N/A
Plasmid: ecDHFR(R12Y, G67S, Y100I)-UBA5(sgResistant)	This study	N/A
Plasmid: NEMF WT (sgResistant)	(Thrun et al., 2021)	N/A
Plasmid: NEMF DR mutant (sgResistant)	(Thrun et al., 2021)	N/A
Plasmid: Cyto-VV-K0	This study	N/A
Plasmid: Cyto-VV-K20	This study	N/A
Plasmid: SS-VV-K0	This study	N/A
Plasmid: SS-VV-K20	This study	N/A
Plasmid: SS-VgV-K0	This study	N/A
Plasmid: SS-VgV-K20	This study	N/A
Plasmid: SS-gVgV-K20	This study	N/A
Plasmid: TM-VgV-K0	This study	N/A
Plasmid: TM-VgV-K20	This study	N/A
Plasmid: PPL-K0	This study	N/A
Plasmid: PPL-K20	This study	N/A
Plasmid: SS-GFP-L	This study	N/A
Plasmid: SS-GFP-S	This study	N/A
Software and Algorithms		
Image Studio Lite version 5.2.5	LI-COR Biosciences	https://www.licor.com/bio/image-studio/
Prism 9	GraphPad	https://www.graphpad.com/scientific-software/prism/
SnapGene	Dotmatics	https://www.snapgene.com/
Other		
Trans-Blot Turbo RTA Midi NitrocelluloseTransfer Kit	Bio-Rad Laboratories	1704271
4-20% Mini-PROTEAN TGX Gel 10 well	Bio-Rad Laboratories	4561094
4-20% Mini-PROTEAN TGX Gel 15 well	Bio-Rad Laboratories	4561096