

TOLLIP acts as a cargo adaptor to promote lysosomal degradation of aberrant ER membrane proteins

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Abstract

Endoplasmic reticulum (ER) proteostasis is maintained by various catabolic pathways. Lysosomes clear entire ER portions by ER-phagy, while proteasomes selectively clear misfolded or surplus aberrant proteins by ER-associated degradation (ERAD). Recently, lysosomes have also been implicated in the selective clearance of aberrant ER proteins, but the molecular basis remains unclear. Here, we show that the phosphatidylinositol-3-phosphate (PI3P)-binding protein TOLLIP promotes selective lysosomal degradation of aberrant membrane proteins, including an artificial substrate and motoneuron disease-causing mutants of VAPB and Seipin. These cargos are recognized by TOLLIP through its misfolding-sensing intrinsically disordered region (IDR) and ubiquitin-binding CUE domain. In contrast to ER-phagy receptors, which clear both native and aberrant proteins by ER-phagy, TOLLIP selectively clears aberrant cargos by coupling them with the PI3P-dependent lysosomal trafficking without promoting bulk ER turnover. Moreover, TOLLIP depletion augments ER stress after ERAD inhibition, indicating that TOLLIP and ERAD cooperatively safeguard ER proteostasis. Our study identifies TOLLIP as a unique type of cargo-specific adaptor dedicated to the clearance of aberrant ER cargos and provides insights into molecular mechanisms underlying lysosome-mediated quality control of membrane proteins.

Keywords cargo adaptor; ER-phagy; intrinsically disordered region; lysosome; TOLLIP

Subject Categories Autophagy & Cell Death; Post-translational Modifications & Proteolysis

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Introduction

Lysosomes play essential roles in degrading and recycling proteins, lipids, nucleic acids, and other biomaterials. These cargos reach

lysosomes through diverse trafficking routes. For example, cytoplasmic materials such as organelles and proteins are sequestered and delivered to lysosomes mainly by autophagosomes, which is referred to as macroautophagy (Morishita & Mizushima, 2019). Since most cargos are transferred to lysosomes via large vesicles, lysosomal degradation can be nonselective, as exemplified by the starvation-induced bulk macroautophagic degradation of cytoplasmic materials. However, lysosomal degradation can also be selective. Selectivity is conferred by cargo adaptors tethering cargos to components of membrane-trafficking machinery (Gatica *et al*, 2018; Sigismund *et al*, 2021). Examples include autophagy receptors that bind to cytoplasmic organelles or proteins and to ATG8 family proteins via their LC3-interacting regions (LIRs) (Gatica *et al*, 2018). ATG8 proteins are covalently attached to growing isolation membranes, which enables selective engulfment of cargos recruited by autophagy receptors into autophagosomes.

Misfolded cytosolic proteins, especially proteasome-resistant aggregates, are typical substrates of cargo adaptor-mediated lysosomal degradation. Aggregation-prone substrates are sorted for macroautophagy through adaptor proteins called aggrephagy receptors, including p62 (Pankiv *et al*, 2007), NBR1 (Kirkin *et al*, 2009), OPTN (Korac *et al*, 2013), TOLLIP (Lu *et al*, 2014), TAX1BP1 (Sarraf *et al*, 2020), and CCT2 (Ma *et al*, 2022). In this aggrephagy pathway, cargos are recruited to ATG8 proteins on isolation membranes by aggrephagy receptors harboring cargo-binding domains that recognize ubiquitin chains or misfolded regions of cargos and LIRs.

In contrast to cytosolic proteins, secretory and membrane proteins accumulate in the endoplasmic reticulum (ER) when they misfold during biogenesis (Sun & Brodsky, 2019). The best-characterized degradation pathway for these proteins is the ER-associated degradation (ERAD) pathway, in which substrates are transported into the cytosol for clearance by the ubiquitin–proteasome system (Christianson & Carvalho, 2022). However, an increasing number of aberrant ER proteins have been reported to be degraded by lysosomes (Reggiori & Molinari, 2022).

The major lysosomal degradation pathway for aberrant secretory proteins is ER-phagy, the bulk lysosomal degradation of ER

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fragments. In ER-phagy, ER fragments are engulfed by autophagosomes (macro-ER-phagy) or directly engulfed by or fused with endolysosomes (micro-ER-phagy and vesicular transport, respectively) with the aid of LIR-containing adaptors termed ER-phagy receptors (Chino & Mizushima, 2020; Reggiori & Molinari, 2022). Although constituents of the ER are cleared as a whole in ER-phagy, several luminal cargos are selectively recognized and cleared by ER-phagy receptors. The ER-phagy receptors FAM134B and RTN3 interact with Calnexin that recognizes α 1-antitrypsin Z (ATZ) and misfolded procollagen and with PGRMC1 that recognizes mutant pro hormones, respectively, to clear these specific cargos (Fregno *et al*, 2018; Cunningham *et al*, 2019; Forrester *et al*, 2019; Chen *et al*, 2021). Another ER-phagy receptor, CCPG1, directly recognizes ER luminal cargos to drive their efficient removal (Ishii *et al*, 2023). ER-phagy receptors act as cargo adaptors to target specific luminal cargos to lysosomal degradation in these cases.

In addition to secretory proteins, various aberrant membrane proteins are degraded by lysosomes through ER-phagy or post-ER quality control pathways (Reggiori & Molinari, 2022). ER chaperones such as DNAJB12 (He *et al*, 2021; Kennedy *et al*, 2022), Calnexin, Calreticulin, and protein disulfide isomerase family A member 3 (PDIA3) (Zhang *et al*, 2022a) have been implicated in the recognition and lysosomal degradation of aberrant membrane proteins. However, it remains a mystery how cargo recognition and lysosomal delivery are coupled for the clearance of aberrant membrane proteins, since cargo adaptors that connect membrane protein cargos with lysosomal trafficking machinery have not been identified. Moreover, whether there exist dedicated cargo adaptors that promote lysosomal delivery of specific ER cargos but not the bulk ER remains uncertain.

In this study, we identified TOLLIP as a cargo adaptor that recognizes and targets aberrant membrane proteins for lysosomal degradation. Unlike its role as the LC3-binding aggrephagy receptor, TOLLIP couples membrane protein cargos with phosphatidylinositol-3-phosphate (PI3P)-dependent lysosomal trafficking machinery. In striking contrast to ER-phagy receptors, TOLLIP is not involved in bulk ER turnover but promotes selective clearance of aberrant ER cargos, representing an enticing therapeutic target for diseases involving the accumulation of aberrant membrane proteins.

Results

Lysosomal degradation of a model aberrant ER membrane protein

Most of the aberrant membrane proteins that have been reported to be degraded by lysosomes are multipass membrane proteins, including GnRHR and NPC1 mutants (Houck *et al*, 2014; Schultz *et al*, 2018). Similar to ERAD, the process for the degradation of these topologically complex substrates is likely complicated, engaging multiple and redundant quality control factors (Bernasconi *et al*, 2010). To overcome likely complications due to this predicted complexity, we developed an artificial aberrant ER membrane protein, A53T-HP, which is a chimeric protein with simple structure consisting of the Parkinson's disease-linked A53T α -synuclein mutant and the hairpin region of the ER membrane protein E-Syt1

(Fig 1A). Since cytosolic A53T α -synuclein is efficiently degraded by lysosomes (Webb *et al*, 2003), we reasoned that A53T-HP behaves as an aberrant ER membrane protein with a cytosolic degron, facilitating its lysosomal degradation. A53T-HP exhibited a reticular ER pattern (Fig 1B and Appendix Fig S1A) and was cofractionated with E-Syt1 (Fig 1C), confirming its ER localization. The lysosome inhibitor bafilomycin A1 significantly increased A53T-HP, but not the native ER membrane protein E-Syt1, indicating that A53T-HP was selectively degraded by lysosomes as an aberrant protein (Fig 1D). Lysosomal degradation of specific cargos can be specifically and quantitatively monitored by the fluorescent protein-based processing assay (Mizushima *et al*, 2010; Liang *et al*, 2018; Chino & Mizushima, 2020). For example, ER-phagy activity can be monitored by the proteolytic cleavage of the lysosome-resistant RFP fragment from the ER luminal tandem RFP-EGFP reporter (Chino *et al*, 2019) (Appendix Fig S1B). Similar to this reporter, the cleavage of mCherry, a variant of RFP, from mCherry-tagged A53T-HP was detected, which was impaired by bafilomycin A1 but not by proteasome inhibitors (Fig 1E). Taken together, these properties validated A53T-HP as an integral ER membrane substrate destined for lysosomal degradation.

TOLLIP promotes lysosomal degradation of an aberrant ER membrane protein

To explore the mechanism involved in the lysosomal degradation of A53T-HP, we first assessed the involvement of aggrephagy receptors, which promote the lysosomal degradation of aberrant cytosolic proteins such as polyQ-expanded Huntingtin (Gatica *et al*, 2018). When overexpressed, TOLLIP most strongly promoted the cleavage of lysosomal degradation-resistant mCherry from mCherry-A53T-HP (Fig 2A). In contrast, siRNA-mediated knockdown of TOLLIP impaired mCherry-A53T-HP processing in HEK293A and HeLa cells, while the effect of knocking down other receptors was smaller (Figs 2B, and EV1A and B). A previous study had reported that TOLLIP was not involved in regulating global lysosomal degradation activity (Lu *et al*, 2014). Similarly, we found that depletion of TOLLIP did not increase the expression level of the lysosomal substrate p62 (Fig 2B). The trafficking of mCherry-EGFP-tagged A53T-HP to RAB7-positive endolysosomes and subsequent quenching of the EGFP signal were diminished by TOLLIP knockdown, confirming that TOLLIP was required for lysosomal delivery of A53T-HP (Figs 2C and EV1C). Importantly, TOLLIP overexpression enhanced the lysosomal degradation of A53T-HP as examined by mCherry processing, but not that of cytosolic A53T α -synuclein or native E-Syt1, suggesting cargo selectivity of TOLLIP (Fig EV1D).

In addition to its role as an aggrephagy receptor, TOLLIP has been reported to regulate the endosomal sorting of internalized cell surface proteins (Brissoni *et al*, 2006) and mitochondrial-derived vesicles (MDVs) (Li *et al*, 2021). TOLLIP harbors three domains, including the TOM1-binding domain (TBD), which is involved in the endosomal sorting via interaction with the endosomal protein TOM1, the phosphoinositide-binding C2 domain, which also harbors two tandem LIRs, and the ubiquitin-binding CUE domain. Since the region between the C2 and CUE domains was predicted to be highly disordered, we termed it the intrinsically disordered region (IDR) (Fig 2D). We investigated whether each domain in TOLLIP is required for the lysosomal degradation of A53T-HP. mCherry-A53T-HP

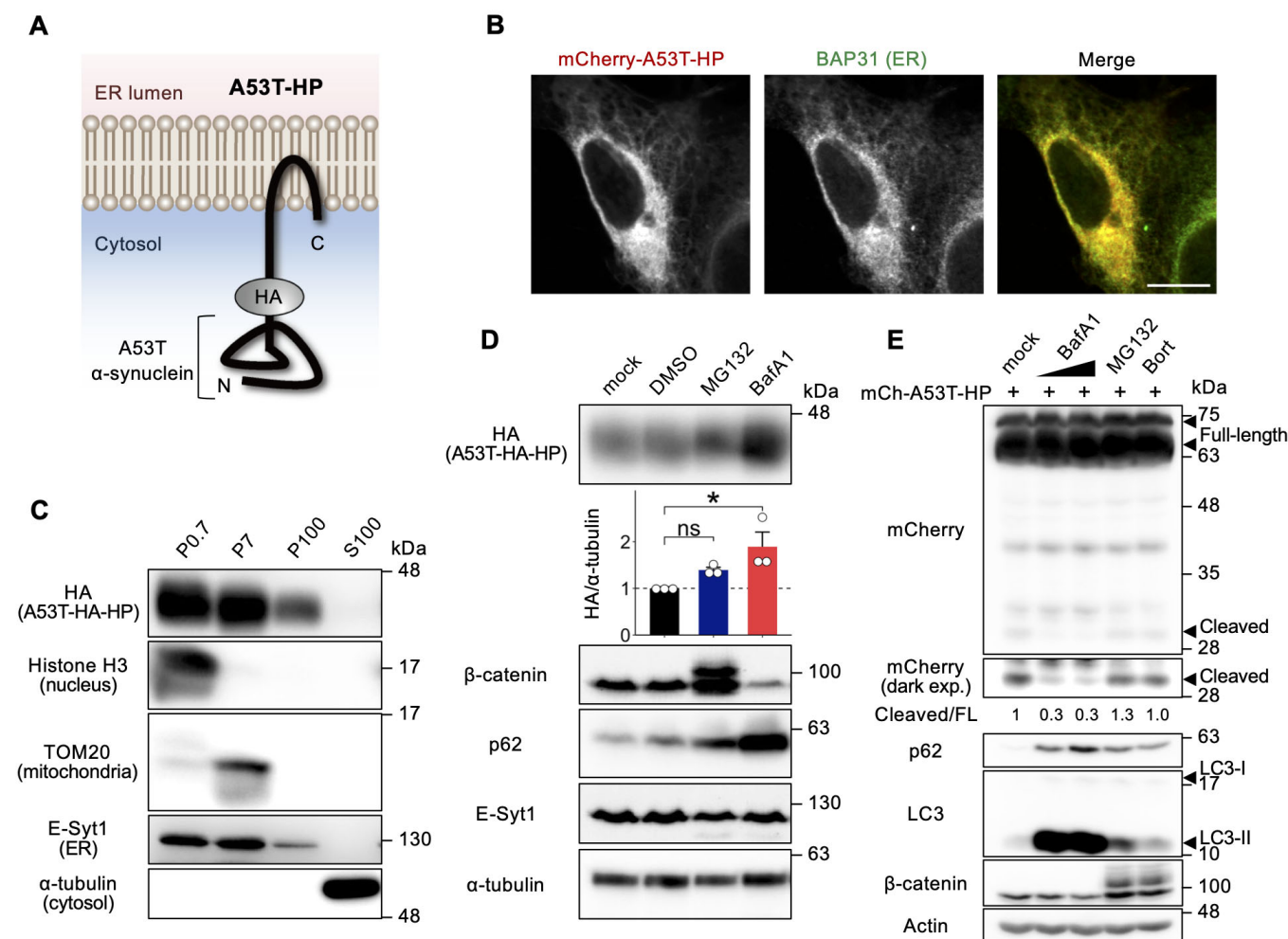


Figure 1. Lysosomal degradation of a model aberrant ER membrane protein.

A Schematic representation of the artificial model substrate A53T-HP used in this study. The C-terminal of A53T α -synuclein is fused with an HA tag (or a FLAG tag in Fig 3A) and the hairpin region of E-Syt1.

B Immunofluorescence of HEK293A cells expressing tetracycline-inducible mCherry-A53T-HP stained with an anti-BAP31 antibody. Scale bar, 10 μ m (n = 2 independent experiments).

C Differential centrifugation of HEK293 cells expressing tetracycline-inducible A53T-HP. P0.7, nuclear fraction; P7, mitochondrial fraction; P100, microsomal fraction; S100, cytosolic fraction (n = 3 independent experiments).

D Immunoblot of HEK293 cells expressing tetracycline-inducible A53T-HP treated with 5 μ M MG132 or 100 nM bafilomycin A1 (BafA1) for 20 h. The graph represents mean $\pm$ SEM of the A53T-HP level normalized to α -tubulin (n = 3 independent experiments). β -catenin and p62 served as positive controls for proteasome and lysosome inhibition, respectively. * P < 0.05 by one-way ANOVA with Dunnett's multiple comparison test.

E Lysosomal delivery of A53T-HP monitored by mCherry processing. HEK293A cells transfected with mCherry-A53T-HP were treated with increasing concentrations of BafA1 (200 or 400 nM), 5 μ M MG132, or 500 nM Bortezomib (Bort) for 24 h. Numerical data represent means of the ratio of cleaved mCherry:full-length (FL) mCherry-A53T-HP (n = 3 independent experiments). Doublets of mCherry-A53T-HP may represent two distinct conformational states of the mCherry tag, which is often observed in SDS-PAGE of fluorescent protein-tagged membrane proteins (Geertsma *et al*, 2008; Houck *et al*, 2014). p62 and LC3 served as positive controls for lysosome inhibition. LC3-I, the unlipidated form of LC3; LC3-II, the lipidated form of LC3.

Source data are available online for this figure.

processing was impaired by *TOLLIP* knockout (KO), and re-expression of wild-type (WT) *TOLLIP* or a TOM1-binding-defective TBD mutant (Xiao *et al*, 2015) restored mCherry processing (Fig 2E). In contrast, mCherry processing was not restored in cells expressing mutants with deleted C2, IDR, or CUE domains, an IDR mutant in which the IDR was replaced with an artificial flexible linker, or a ubiquitin binding-defective CUE mutant (Lu *et al*, 2014) (Fig 2E). Notably, mCherry processing was restored by the LIR mutant, but not by the C2

mutant R123A (mut #1), which is defective in phosphoinositide binding (Ankem *et al*, 2011) (Fig 2F). The C2, IDR, and CUE domains were also required for the *TOLLIP*-dependent delivery of A53T-HP to LAMP1-positive endolysosomes (Fig 2G) and for the turnover of A53T-HP as examined by pulse-chase assays (Fig EV1E and F). Overall, these data suggest that phosphoinositide binding through the C2 domain, a previously uncharacterized function of the IDR, and ubiquitin binding through the CUE domain are required for A53T-HP

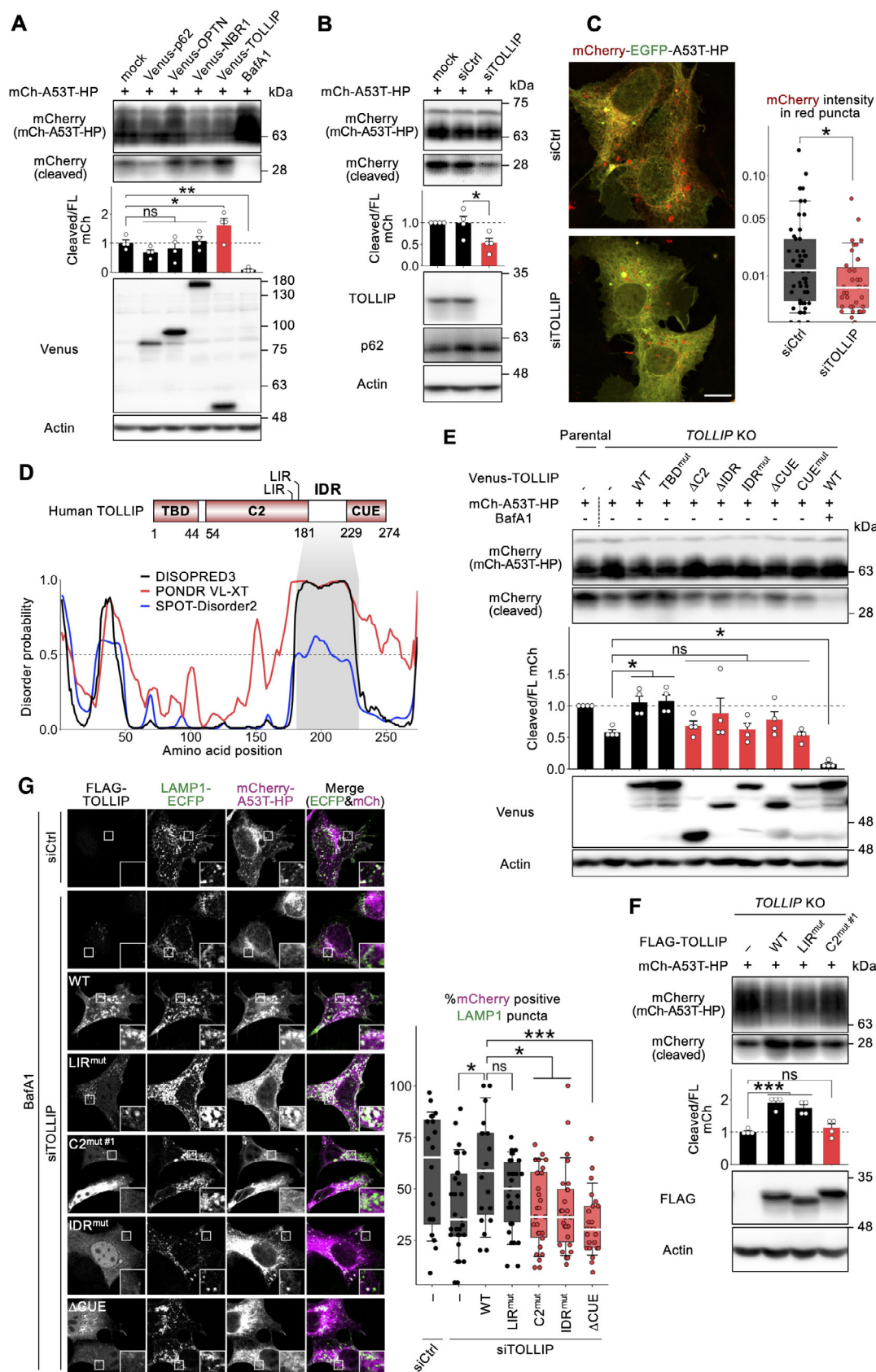


Figure 2.

Figure 2. TOLLIP promotes lysosomal degradation of an aberrant ER membrane protein.

- A Lysosomal degradation of A53T-HP in HEK293A cells transfected with Venus-tagged aggrephagy receptors and mCherry-A53T-HP. The graph represents mean $\pm$ SEM of mCherry processing ($n = 4$ independent experiments).
- B Lysosomal degradation of A53T-HP in HEK293A cells transfected with indicated siRNAs and mCherry-A53T-HP. The graph represents mean $\pm$ SEM of mCherry processing ($n = 4$ independent experiments).
- C Fluorescence microscopy imaging to monitor the lysosomal delivery of A53T-HP. HEK293A cells were transfected with indicated siRNAs and mCherry-EGFP-A53T-HP. The mCherry intensity in red puncta relative to the total mCherry intensity in each cell was quantified. 56 (siCtrl) or 39 (siTOLLIP) cells pooled from $n = 3$ independent experiments were analyzed. Scale bar, 10 μ m.
- D Domain architecture of TOLLIP. IDR was predicted through structural predictions.
- E, F Lysosomal degradation of A53T-HP in parental or TOLLIP-KO HEK293A cells transfected with TOLLIP variants and mCherry-A53T-HP. The graph represents mean $\pm$ SEM of mCherry processing ($n = 4$ independent experiments). TBD^{mut}, F21A; Δ C2, Δ 53–178; Δ IDR, Δ 181–229; IDR^{mut}, 181–229 > (GGGGG)₅GGGG; Δ CUE, Δ 231–274; CUE^{mut}, L267A and L268A; LIR^{mut}, W133A, T134A, H135A, I136A, W151A, Y152A, S153A, and L154A; C2^{mut} #1, R123A.
- G Fluorescence microscopy imaging to monitor the lysosomal delivery of A53T-HP. HEK293A cells were transfected with indicated siRNAs, FLAG-TOLLIP variants, LAMP1-ECFP, and mCherry-A53T-HP, treated with 100 nM BafA1 for 19 h, and stained with an anti-FLAG antibody. The percentage of LAMP1 puncta containing mCherry-A53T-HP in each cell was quantified. From 19 to 30 cells in each condition pooled from $n = 2$ independent experiments were analyzed. Scale bars, 10 and 2 μ m (insets).

Data information: Box centers indicate medians, box edges represent first and third quartiles, and whiskers show the 10th to 90th percentile. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by one-way ANOVA with Dunnett's multiple comparison test (A and E–G), unpaired two-tailed Student's *t* test (B), or one-sided Wilcoxon rank sum test (C). Source data are available online for this figure.

clearance. Dispensability of the TBD and LIRs implies that the TOLLIP mode of action in A53T-HP clearance is different from that of other reported TOLLIP functions, including endosomal sorting and aggrephagy.

The TOLLIP IDR and CUE domains coordinately recognize an aberrant ER membrane protein

In the postnuclear membrane fraction, A53T-HP interacted with endogenous TOLLIP, suggesting that TOLLIP recognized A53T-HP on the ER surface (Fig 3A). Through its CUE domain, TOLLIP has been reported to recognize the ubiquitin chains of cytosolic polyQ aggregates and endocytosed cell surface proteins (Brissoni *et al*, 2006; Lu *et al*, 2014). To map the regions of TOLLIP required for A53T-HP recognition, we assessed the interaction between A53T-HP and a series of TOLLIP mutants in TOLLIP-KO cells. Consistent with the reported substrate-binding role, the interaction between A53T-HP and TOLLIP was reduced by deletion or point mutations in the CUE domain (Fig 3B and C). Unexpectedly, mutations in the IDR also led to defective TOLLIP binding to A53T-HP (Fig 3B). Although deletion of the C2 domain partially impaired the interaction of TOLLIP with A53T-HP, phosphoinositide binding-defective R123A and R78A C2 mutants (Ankem *et al*, 2011) retained the interaction, suggesting that TOLLIP can recognize A53T-HP independently of its phosphoinositide binding (Fig 3B and C). These results suggest that the IDR and CUE domains play substrate recognition roles in A53T-HP degradation.

We further investigated the roles of the IDR and CUE domains in substrate recognition by sequential immunoprecipitation. The interaction between TOLLIP and ubiquitinated species of A53T-HP was abolished by the CUE domain mutation (Fig 3D, top panel). Notably, TOLLIP also interacted with nonubiquitinated species of A53T-HP, and an IDR mutation disrupted the interaction between TOLLIP and both the ubiquitinated and this unmodified species of A53T-HP (Fig 3D, top and second panels). These results suggest that the CUE domain recognizes the ubiquitin chains of A53T-HP, while the IDR recognizes the substrate itself. Indeed, the binding of TOLLIP to ubiquitin conjugates was completely abolished by deletion of the CUE domain, but not by mutations in the IDR, supporting the

functional difference between these two regions (Fig EV2A). Since α -synuclein is an unfolded protein, we speculated that the TOLLIP IDR detects unfolding or misfolding of clients. To test this possibility, we performed an *in vitro* binding assay using heat-denatured luciferase as a model of an aberrant protein recognized by chaperones such as HSP70 (Hjerpe *et al*, 2016) (Fig EV2B). Recombinant GST-tagged TOLLIP IDR but not the CUE domain interacted with heat-denatured luciferase, suggesting that the IDR directly recognized the aberrant folding status of clients (Fig 3E). Overall, these data suggest that TOLLIP interacts with clients by recognizing aberrant folding status through its IDR and ubiquitin chains through its CUE domain.

Ubiquitination enhances lysosomal degradation of an aberrant ER membrane protein

Although the TOLLIP IDR can interact with unmodified A53T-HP, the requirement of the CUE domain for A53T-HP degradation suggests that ubiquitinated species of A53T-HP are more actively degraded. As expected, bafilomycin A1 treatment induced preferential accumulation of ubiquitinated A53T-HP (Fig EV2C). Among several E3 ubiquitin ligases critical for the ubiquitination of α -synuclein, we found that NEDD4 was required for the observed bafilomycin A1-dependent increase in ubiquitinated species (Tofaris *et al*, 2011; Stefanis *et al*, 2019) (Fig EV2C). Moreover, overexpressed NEDD4 promoted the ubiquitination and processing of mCherry-A53T-HP in a ligase activity-dependent manner (Fig EV2D). These data suggest that ubiquitination of clients enhances their degradation, which is consistent with a recent report showing E3 ligase RNF185-dependent lysosomal degradation of Ebola virus glycoprotein GP_{1,2} (Zhang *et al*, 2022a).

TOLLIP promotes PI3P-dependent lysosomal trafficking of an aberrant ER membrane protein

The C2 domain in TOLLIP preferentially interacts with PI3P and PI(4,5)P₂ (Ankem *et al*, 2011). Between these phosphoinositides, PI3P is notably enriched in transport vesicles such as endosomes and autophagosomes destined for fusion with lysosomes (Nascimbeni

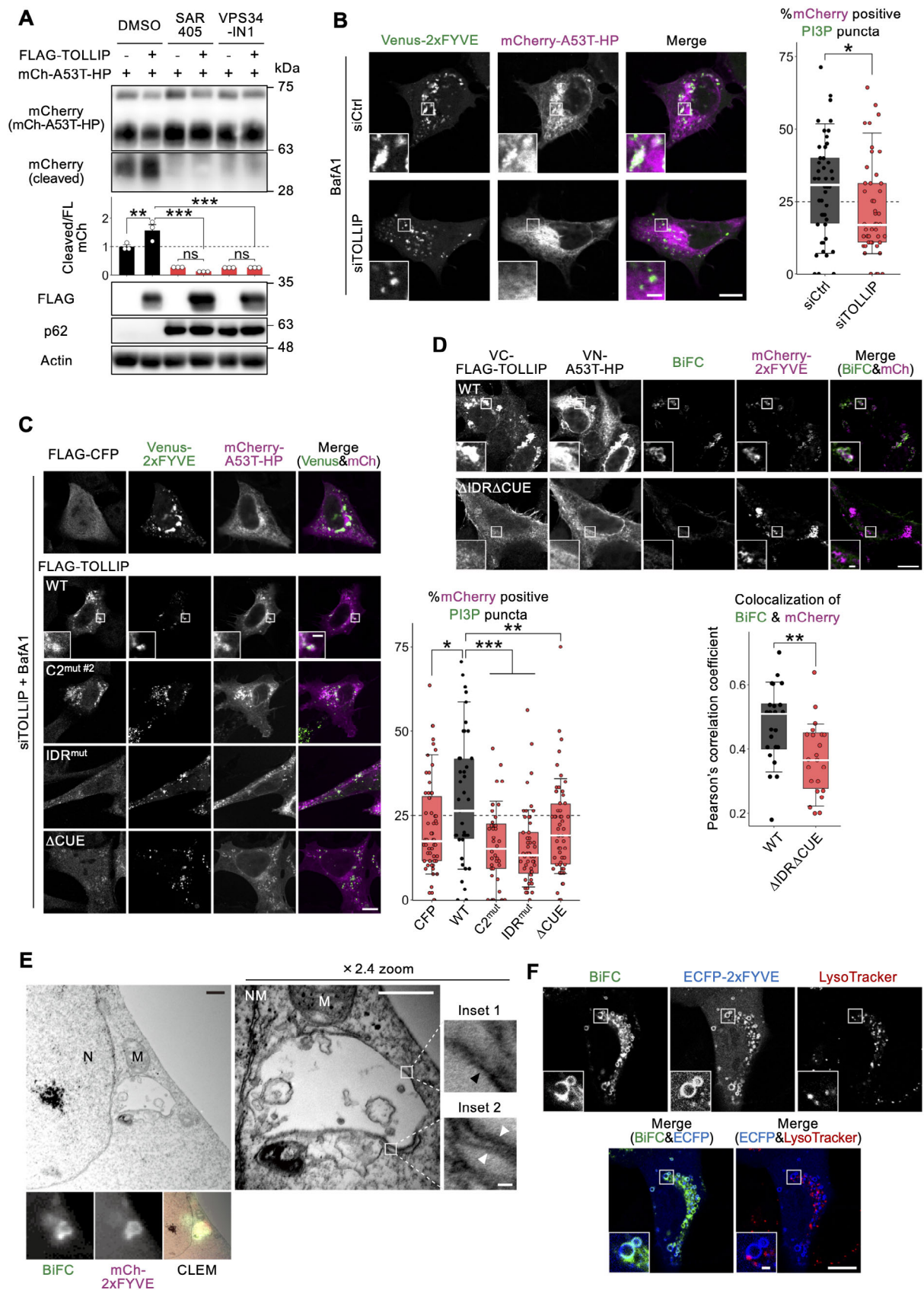


Figure 4.

Figure 4. TOLLIP promotes PI3P-dependent lysosomal trafficking of an aberrant ER membrane protein.

- A Lysosomal degradation of A53T-HP in HEK293A cells transfected with FLAG-TOLLIP and mCherry-A53T-HP and treated with 15 μ M SAR405 or 20 μ M VPS34-IN1 for 24 h. The graph represents mean $\pm$ SEM of mCherry processing ($n = 3$ independent experiments).
- B Fluorescence microscopy imaging to monitor the trafficking of A53T-HP to PI3P-enriched puncta. HEK293A cells were transfected with indicated siRNAs, Venus-2xFYVE, and mCherry-A53T-HP, and treated with 100 nM BafA1 for 16 h. The percentage of 2xFYVE puncta containing mCherry-A53T-HP in each cell was quantified. Forty-five cells in each condition pooled from $n = 3$ independent experiments were analyzed. Scale bars, 10 and 2 μ m (insets).
- C As in (B), but cells were also transfected with indicated siRNA-resistant FLAG-tagged constructs and stained with an anti-FLAG antibody. From 40 to 60 cells in each condition pooled from $n = 3$ independent experiments were analyzed. C2^{mut} #2, R78A.
- D Fluorescence microscopy imaging to monitor the trafficking of the TOLLIP-A53T-HP complex as visualized by BiFC to PI3P-enriched puncta. HEK293A cells were transfected with VC (156–239 aa of mVenus)-FLAG-TOLLIP variants, VN (1–155 aa of mVenus)-A53T-HP, and mCherry-2xFYVE, and stained with anti-FLAG and HA antibodies. The graph represents Pearson's correlation coefficient measured between BiFC signal and mCherry-2xFYVE. A total of 23 (WT) or 22 (Δ IDR Δ CUE) cells pooled from $n = 2$ independent experiments were analyzed. Scale bars, 10 and 1 μ m (insets).
- E CLEM images of HEK293A cells transfected with VC-FLAG-TOLLIP, VN-A53T-HP, and mCherry-2xFYVE. Black arrowhead indicates a single membrane surrounding a vesicle, and white arrowheads indicate a smooth ER-like double-membrane structure attached to the vesicle. N, nucleus; M, mitochondria; NM, nuclear membrane. Scale bars, 500 and 20 nm (insets).
- F Live cell imaging of HEK293A cells transfected with the BiFC constructs as in (E) and ECFP-2xFYVE and stained with LysoTracker. Scale bars, 10 and 1 μ m (insets) ($n = 2$ independent experiments).

Data information: Box centers indicate medians, box edges represent first and third quartiles, and whiskers show the 10th to 90th percentile. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by one-way ANOVA with Tukey's (A) or Dunnett's (C) multiple comparison test, or unpaired two-tailed Student's t test (B and D). Source data are available online for this figure.

was also suppressed by VPS34 knockdown and the VPS34 inhibitor SAR405 (Fig EV3A and B). When the fusion of PI3P-enriched vesicles with lysosomes was blocked by bafilomycin A1 (van Weert *et al*, 1995; Yamamoto *et al*, 1998), A53T-HP accumulated in PI3P-enriched puncta labeled with the 2xFYVE probe (Gillooly *et al*, 2000) (Fig 4B, top panels). Knockdown of TOLLIP suppressed the accumulation of A53T-HP in PI3P puncta, suggesting that TOLLIP was required to transfer A53T-HP from the ER to PI3P puncta (Fig 4B, bottom panels). The accumulation of A53T-HP in PI3P puncta was restored by re-expressing siRNA-resistant WT TOLLIP but not by mutants of the C2, IDR, or CUE domains (Fig 4C). Notably, colocalization of TOLLIP and A53T-HP was detected in PI3P puncta, indicating that TOLLIP escorts A53T-HP to PI3P-enriched vesicles by using these domains (Fig 4C, inset). Supporting this notion, the protein complex of TOLLIP and A53T-HP as visualized by bimolecular fluorescence complementation (BiFC) colocalized with PI3P puncta, which was not observed when TOLLIP lacked the substrate-binding domains IDR and CUE (Fig 4D). Correlative light and electron microscopy (CLEM) analysis revealed that PI3P puncta decorated with the BiFC signal were vesicles surrounded by single membranes (Figs 4E and EV3C, black arrowheads). These vesicles were also partly associated with a smooth ER-like structure (Figs 4E and EV3C, white arrowheads), indicating that they are in close proximity to the ER. Since the BiFC-positive PI3P puncta were not stained by LysoTracker, which identifies acidic compartments, TOLLIP seemed to utilize these PI3P-enriched vesicles as transport intermediates to deliver its cargo to acidic lysosomal compartments (Fig 4F).

TOLLIP is dedicated to clearance of specific ER cargos in contrast to ER-phagy

Several aberrant membrane proteins are targeted to lysosomes after export from the ER to the later secretory compartments (Okuyoneda *et al*, 2010, 2018; Satpute-Krishnan *et al*, 2014; Sun & Brodsky, 2018; Zavodszky & Hegde, 2019; Sun *et al*, 2021). However, the blockade of COPII-mediated ER-Golgi trafficking by H79G SAR1A did not affect either basal or TOLLIP overexpression-dependent

mCherry-A53T-HP clearance (Figs 5A and EV3D). We next tested whether A53T-HP is extracted from the ER membrane to the cytosol by TOLLIP before lysosomal trafficking. However, the accumulation of A53T-HP in the cytosol was not detected even after overexpression of TOLLIP or SAR405 treatment, which impairs the entry of the cytosolic p62 into membranous autophagosomal compartments by impeding autophagosome biogenesis (Fig EV3E). Therefore, A53T-HP seemed to be directly transferred to lysosomes without moving through the secretory pathway or the cytosol.

As a direct ER-to-lysosome delivery route, we focused on ER-phagy. First, we tested whether TOLLIP is an ER-phagy receptor. However, TOLLIP knockdown did not affect mCherry cleavage from ER luminal mCherry-EGFP, a modified version of the ER-phagy reporter developed by Chino *et al* (2019) (Fig 5B). Together with the dispensability of TOLLIP LIRs in A53T-HP degradation (Figs 2F and G, and EV1F), these data collectively suggest that TOLLIP is neither a LIR-containing ER-phagy receptor nor is involved in the lysosomal degradation of the bulk ER. Concordantly, ER constituents were not accumulated in PI3P puncta containing A53T-HP and TOLLIP, suggesting that TOLLIP selectively delivers aberrant cargoes to lysosomes (Fig 5C). To investigate the potential link between A53T-HP degradation and ER-phagy, we next examined whether reported ER-phagy receptors are involved in the lysosomal degradation of A53T-HP. Among the receptors tested, including those involved in the selective clearance of ER luminal cargos (i.e., FAM134B, RTN3, and CCPG1), we found that CCPG1 (Smith *et al*, 2018) was required for the lysosomal degradation of A53T-HP (Fig 5D, and Appendix Fig S2A and B). In contrast to TOLLIP, depletion of CCPG1 significantly suppressed ER-phagy activity, suggesting that CCPG1 promoted lysosomal degradation of a plethora of ER-localized proteins through ER-phagy and that A53T-HP was included in this protein set (Fig 5D). Even in CCPG1-KO cells, mCherry-A53T-HP processing was enhanced by TOLLIP overexpression and reduced by knockdown as well as in parental cells (Figs 5E and EV3F). These data suggest that TOLLIP-dependent ER-to-lysosome delivery and bulk ER-phagy operate, at least partially, in parallel for A53T-HP clearance. In addition, TOLLIP-dependent A53T-HP clearance was not impaired even when autophagosome biogenesis was blocked,

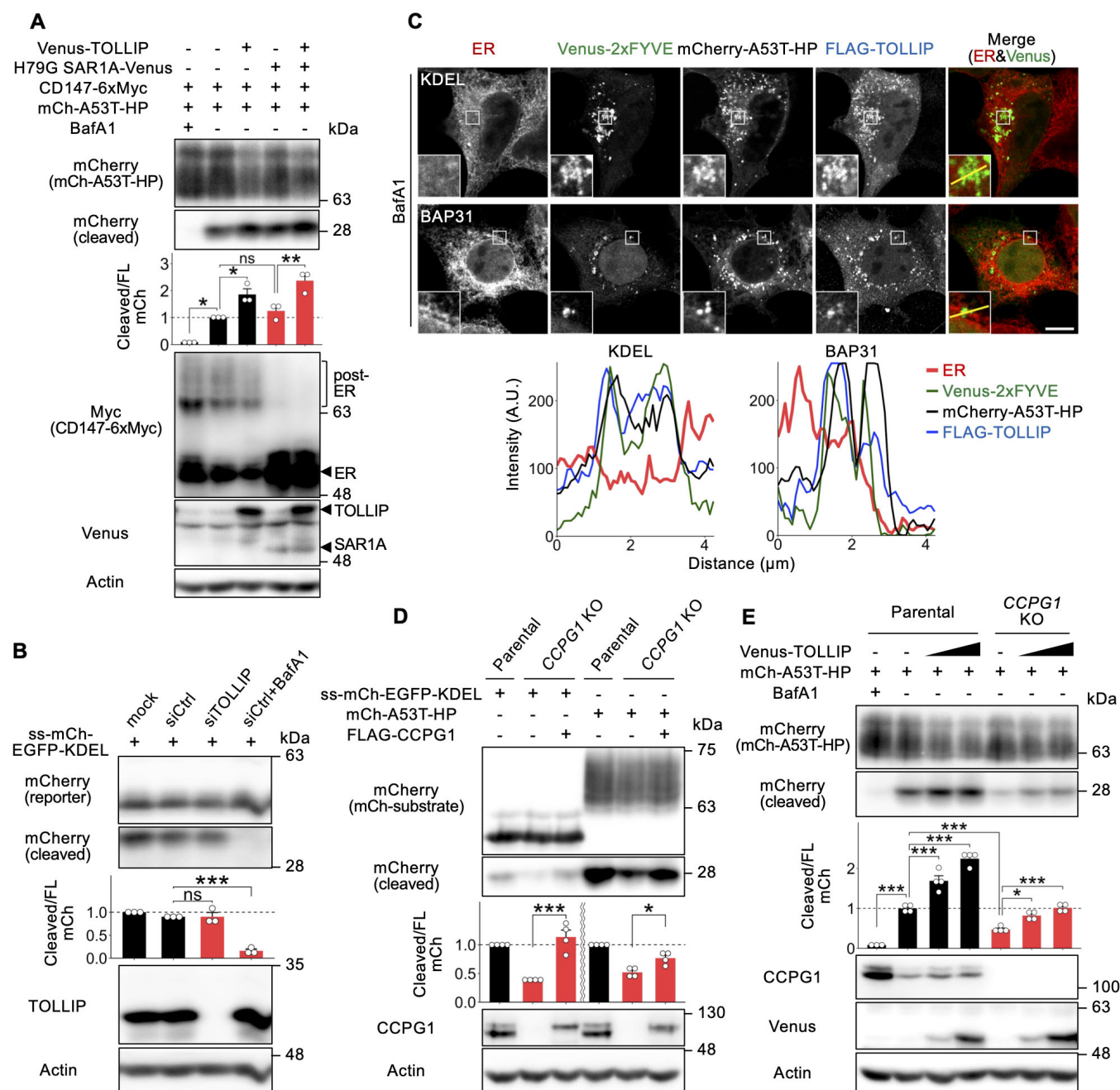


Figure 5. TOLLIP is dedicated to clearance of specific ER cargos in contrast to ER-phagy.

- A** Lysosomal degradation of A53T-HP in HEK293A cells transfected with indicated constructs. The graph represents mean $\pm$ SEM of mCherry processing ($n = 3$ independent experiments). CD147 served to confirm H79G SAR1A-dependent inhibition of the COPII transport (Tyler *et al*, 2012).
- B** ER-phagy activity of HEK293A cells transfected with indicated siRNAs and an ER-phagy reporter. The graph represents mean $\pm$ SEM of mCherry processing ($n = 3$ independent experiments).
- C** Immunofluorescence of HEK293A cells transfected with indicated constructs, treated with 100 nM BafA1 for 15 h, and stained with anti-KDEL or BAP31 antibodies. Line plots show the fluorescence intensities of each channel along the yellow lines. Scale bar, 10 μ m ($n = 2$ independent experiments).
- D, E** ER-phagy activity and lysosomal degradation of A53T-HP in parental or CCPG1-KO HEK293A cells transfected with indicated constructs. The graphs represent mean $\pm$ SEM of mCherry processing ($n = 4$ independent experiments).

Data information: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by one-way ANOVA with Tukey's (A and E) or Dunnett's (B) multiple comparison test, or unpaired two-tailed Student's t test (D).

Source data are available online for this figure.

suggesting that TOLLIP promotes PI3P-dependent and macroautophagy-independent cargo-selective vesicular trafficking of A53T-HP to lysosomes (Fig EV3G–I).

TOLLIP clears disease-linked mutant membrane proteins

Next, we set out to identify physiological clients of TOLLIP. Given that TOLLIP recognizes A53T-HP through the misfolding-sensing IDR and the ubiquitin-binding CUE domain, we speculated that profoundly misfolded and highly ubiquitinated proteins are promising candidates. Among ER-resident membrane proteins that meet these criteria, we identified amyotrophic lateral sclerosis (ALS)-linked P56S VAPB and spastic paraplegia-linked N88S and S90L Seipin as physiological substrates of TOLLIP-dependent lysosomal degradation (Fig 6A and B). These mutants have been suggested to be causative proteins for the respective autosomal dominant motoneuron diseases (Yagi et al, 2011; Tripathi et al, 2021). Consistent with the previous reports (Kanekura et al, 2006; Ito & Suzuki, 2007), these mutants were more heavily ubiquitinated than their WT counterparts (Fig 6C). The abundance of ubiquitinated mutants was further increased by depletion of TOLLIP, but that of WT proteins was only marginally increased, suggesting that TOLLIP prevents the accumulation of misfolded species of mutant membrane proteins (Fig 6C). We next generated HEK293A cell lines stably expressing WT or P56S VAPB to evaluate the expression level of their predominant unmodified species. In these cell lines, TOLLIP knockdown led to the accumulation of P56S VAPB but not WT (Fig 6D). Moreover, the turnover of P56S VAPB, but not WT, was delayed by TOLLIP knockdown, corroborating the selective clearance of misfolded membrane proteins by TOLLIP (Fig EV4A).

Both VAPB and Seipin mutants interacted with endogenous TOLLIP with greater affinity than their respective WT counterparts (Fig 6E). Similar to recognition of A53T-HP, the IDR and CUE domains of TOLLIP were required for the recognition (Fig EV4B and C). A Venus-tagged TOLLIP IDR fragment was sufficient for selective binding to the mutant proteins (Fig 6F). Together with evidence showing direct TOLLIP IDR binding to heat-denatured luciferase (Fig 3E), these results suggest that the TOLLIP IDR is capable of detecting misfolding of various distinctly shaped proteins. Furthermore, P56S VAPB aggregates, which had also been observed in

biopsy samples from an ALS patient carrying this mutation (Tripathi et al, 2021), but not WT VAPB colocalized with TOLLIP (Fig 6G). Overall, these results support the notion that TOLLIP selectively clears misfolded membrane proteins by selectively recognizing them.

Among the clients of TOLLIP, P56S VAPB was insoluble in a non-ionic detergent, while A53T-HP and Seipin mutants were soluble (Fig EV4D). In addition to these clients bearing two or less trans-membrane domains, we also found that TOLLIP expression promoted the lysosomal degradation of the hypogonadotropic hypogonadism-linked E90K GnRHR mutant bearing seven trans-membrane domains, which was previously reported as an aberrant membrane protein destined for lysosomal degradation (Houck et al, 2014) (Fig EV4E). Therefore, TOLLIP promotes the clearance of topologically diverse clients with broad biochemical properties.

Notably, expression of Seipin mutants induced ER stress (Fig EV4D), which is proposed to underlie the pathogenesis of spastic paraplegia caused by Seipin mutations (Ito & Suzuki, 2007; Yagi et al, 2011). In neural SH-SY5Y cells, Seipin mutant-dependent ER stress was suppressed by coexpressing TOLLIP, concomitant with the decrease in the mutant protein level (Fig 7A). In the more physiologically relevant mouse motoneuron-like NSC34 cells, overexpression of mouse Tollip significantly accelerated the degradation of N88S Seipin with little impact on WT Seipin (Figs 7B and EV4F). Collectively, these results suggest that TOLLIP regulates Seipin mutant-dependent ER stress by promoting their clearance in neural cells.

TOLLIP suppresses ER stress after ERAD inhibition

The proteasome-dependent ERAD pathway maintains ER proteostasis through the degradation of functionally and structurally diverse clients (Christianson & Carvalho, 2022). Hence, genetic or pharmacological perturbations of key ERAD factors lead to the accumulation of misfolded proteins in the ER, i.e., ER stress. Since TOLLIP is required for the clearance of various aberrant cargos, we speculated that depletion of TOLLIP might induce ER stress. First, we examined the basal expression levels of the ER stress markers BiP and HERP and found that these levels were not increased by TOLLIP-KO (Fig 8A, 0 h). Then, we treated cells with the VCP inhibitor eeyarestatin I (EerI) at a relatively low concentration, which

Figure 6. TOLLIP clears disease-linked mutant membrane proteins.

- Schematic representation of motoneuron disease-linked mutants of VAPB and Seipin.
- Lysosomal degradation of VAPB and Seipin mutants in HEK293A cells transfected with indicated siRNAs and mCherry-tagged mutants. The graph represents mean $\pm$ SEM of mCherry processing ($n = 3$ independent experiments).
- Denaturing IP to evaluate the abundance of ubiquitinated species of VAPB and Seipin in parental or TOLLIP-KO HEK293A cells. The graph represents mean $\pm$ SEM of the relative amount of ubiquitinated species (top panel) normalized to unmodified species (second panel; $n = 4$ independent experiments).
- Immunoblot of HEK293A cells stably expressing tetracycline-inducible mCherry-VAPB variants treated with indicated siRNAs. Numerical data represent mean $\pm$ SEM of TOLLIP KD-dependent fold change in the mCherry-VAPB level normalized to α -tubulin ($n = 3$ independent experiments).
- Co-IP of VAPB and Seipin variants with endogenous TOLLIP in HEK293A cells. Numerical data represent means of the relative amount of coimmunoprecipitated TOLLIP normalized to precipitated VAPB or Seipin ($n = 3$ independent experiments). Values for each WT protein were normalized to 1.
- Co-IP of VAPB and Seipin variants with Venus-TOLLIP IDR (181–229) in TOLLIP-KO HEK293A cells. Numerical data represent means of the relative amount of coimmunoprecipitated Venus-IDR normalized to its expression level in lysates and precipitated VAPB or Seipin ($n = 3$ independent experiments). Values for each WT protein were normalized to 1.
- Immunofluorescence of HEK293A cells transfected with mCherry-VAPB variants and Venus-TOLLIP and stained with an anti-BAP31 antibody. Scale bars, 10 and 2 μ m (insets) ($n = 3$ independent experiments).

Data information: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by one-way ANOVA with Dunnett's multiple comparison test (B), or unpaired two-tailed Student's t test (C). #, unspecific bands. †, presumed degradation products of Seipin.

Source data are available online for this figure.

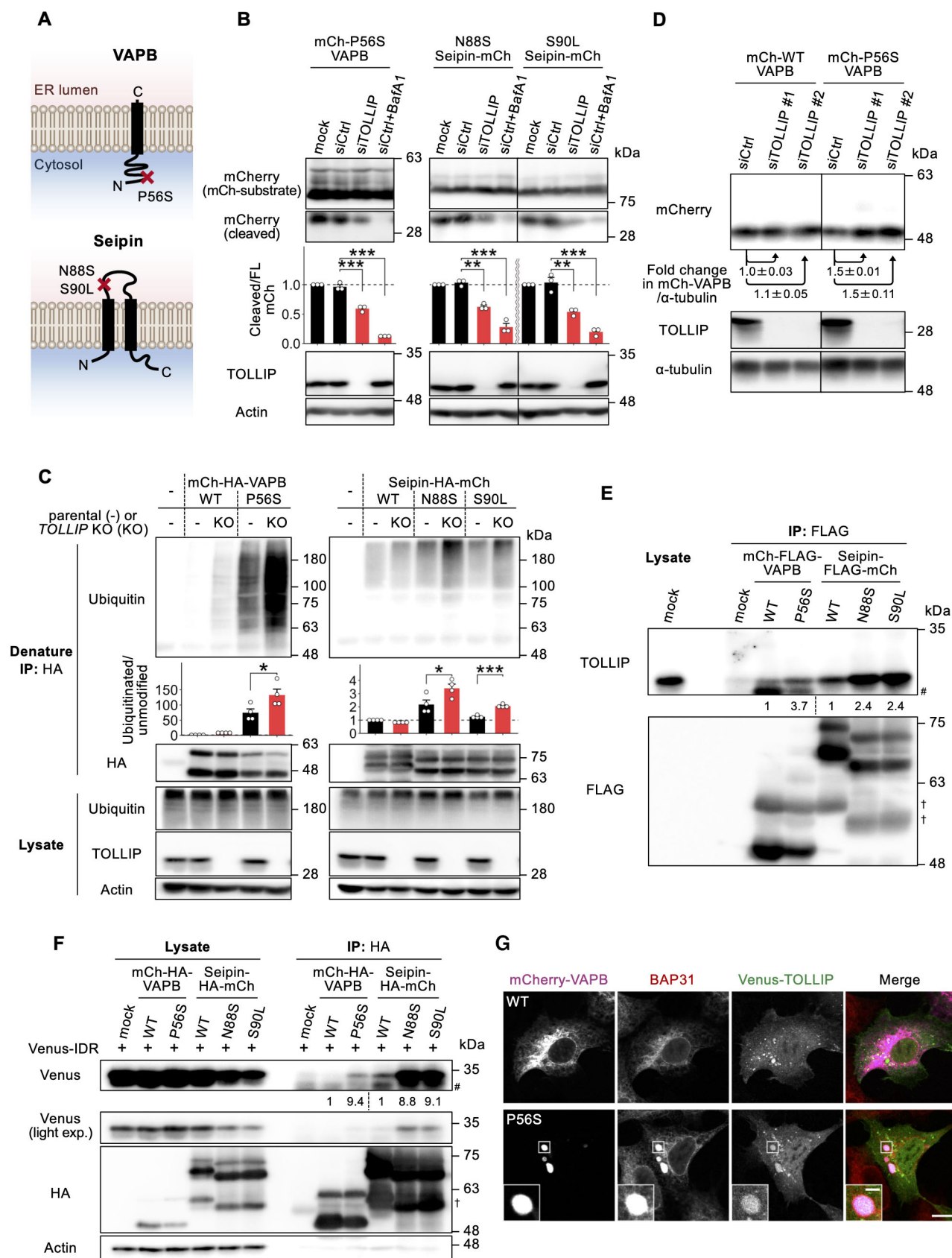


Figure 6.

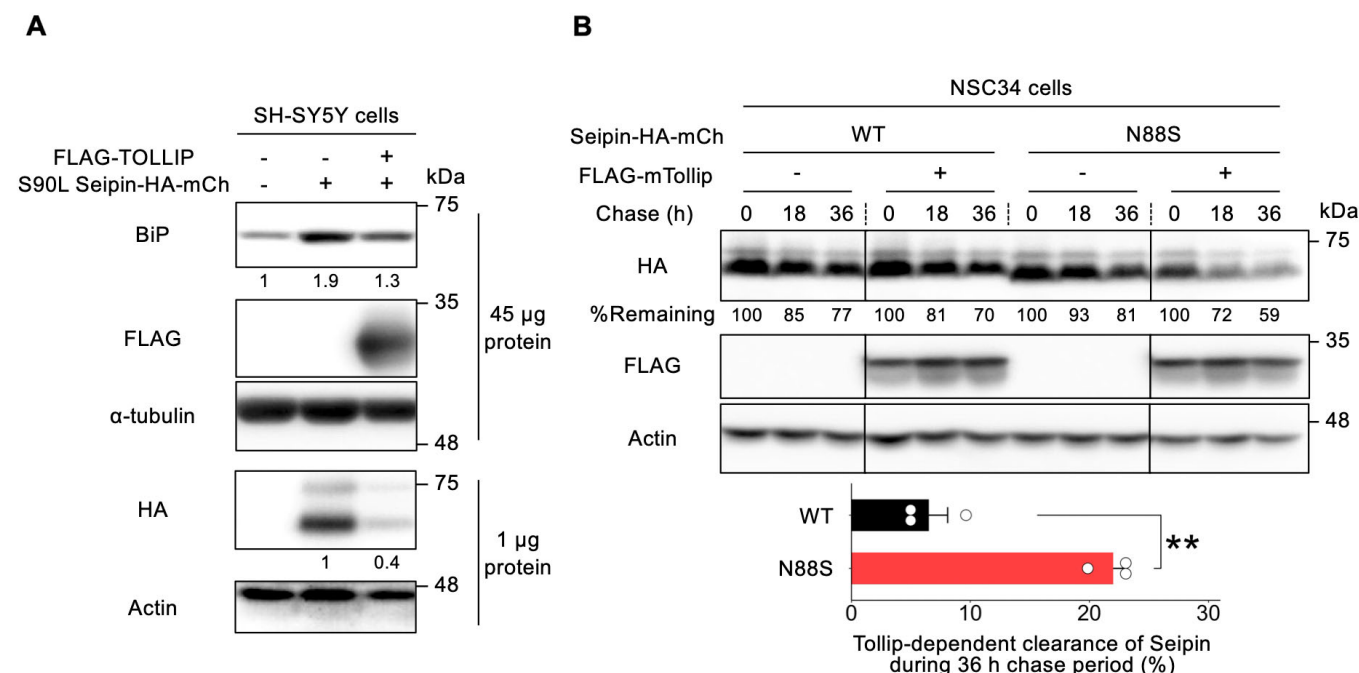


Figure 7. TOLLIP clears motoneuron disease-causing mutant membrane proteins in neural cells.

A Immunoblot of SH-SY5Y cells transfected with an equal amount of plasmid encoding S90L Seipin-HA-mCherry with or without FLAG-TOLLIP. Equal amounts of proteins were loaded on different gels to probe indicated proteins. Numerical data represent means of the relative amount of the ER stress marker BiP and S90L Seipin-HA-mCherry ($n = 3$ independent experiments).

B Turnover of Seipin variants monitored by promoter shut-off assay. NSC34 cells were transfected with HA-tagged Seipin variants harboring a tetracycline (Tet)-responsive promoter and tetracycline-controlled transactivator (tTA). After 24 h, cells were treated with 2.5 μ g/ml Tet to halt the transcription of Seipin variants. Twenty-four hours after Tet addition was set to time 0, and the remaining Seipin protein level was evaluated at indicated time points. Numerical data represent the percentage of remaining Seipin at each time point, and the graph represents mean $\pm$ SEM of the percentage of Seipin cleared by Tollip expression during the chase period ($n = 3$ independent experiments). ** $P < 0.01$ by unpaired two-tailed Student's t test.

Source data are available online for this figure.

induces the gradual, but not acute, accumulation of misfolded proteins in the ER through ERAD inhibition (Wang *et al*, 2009) (Fig EV5A–C). Under this condition, BiP and HERP were gradually increased, which was further enhanced by TOLLIP-KO (Fig 8A). Consistently, transcripts of ER stress markers were increased by TOLLIP-KO not in the basal state but after EerI treatment, which was suppressed by stably re-expressing Venus-TOLLIP (Figs 8B and EV5D). Furthermore, when ERAD was inhibited by another VCP inhibitor NMS-873 or proteasome inhibitors, ER stress monitored by the induction of BiP and splicing of *XBPI* mRNA was also augmented in TOLLIP-KO cells (Fig 8C). These results suggest that TOLLIP is important for ER proteostasis especially when ERAD, which acts in parallel with the TOLLIP-dependent pathway, is inhibited.

Discussion

An increasing number of mammalian ER-phagy receptors have been identified: FAM134A/B/C (Khaminets *et al*, 2015; Reggio *et al*, 2021), SEC62 (Fumagalli *et al*, 2016), RTN3 (Grumati *et al*, 2017), CCPG1 (Smith *et al*, 2018), ATL3 (Chen *et al*, 2019), TEX264 (An *et al*, 2019; Chino *et al*, 2019), p62 (Ji *et al*, 2019), CALCOCO1

(Nthiga *et al*, 2020), and C53 (Stephani *et al*, 2020). In addition to their primary roles in bulk ER turnover by connecting the ER and lysosomal degradation machinery, some ER-phagy receptors add another layer of specificity by recognizing aberrant proteins among an abundant sea of normal ER-resident proteins. In this study, we identified TOLLIP as another cargo adaptor connecting ER constituents with lysosomal degradation machinery. In striking contrast to the 11 aforementioned ER-phagy receptors, the TOLLIP-dependent lysosomal delivery of ER cargos is unique for sparing the bulk ER and connecting the cargos with PI3P (Fig 8D). Our data suggest that PI3P-dependent non-macroautophagic trafficking is involved in this process. Examples of such ER-to-lysosome trafficking include the vesicular delivery of ATZ (Fregno *et al*, 2018). However, dispensability of the ATG8 conjugation system distinguishes the TOLLIP-dependent pathway from ATZ clearance. Our BiFC data show that A53T-HP moves to PI3P-enriched vesicular transport intermediates after its recognition by TOLLIP (Figs 4D–F and EV3C). The precise route of this TOLLIP-dependent pathway awaits future investigation; especially, it remains to be investigated how aberrant membrane proteins are exported from the ER and whether the PI3P-enriched vesicles fuse with or are engulfed by lysosomes.

In this study, we characterized a novel functional region TOLLIP IDR as a protein misfolding sensor. In addition to A53T-HP and

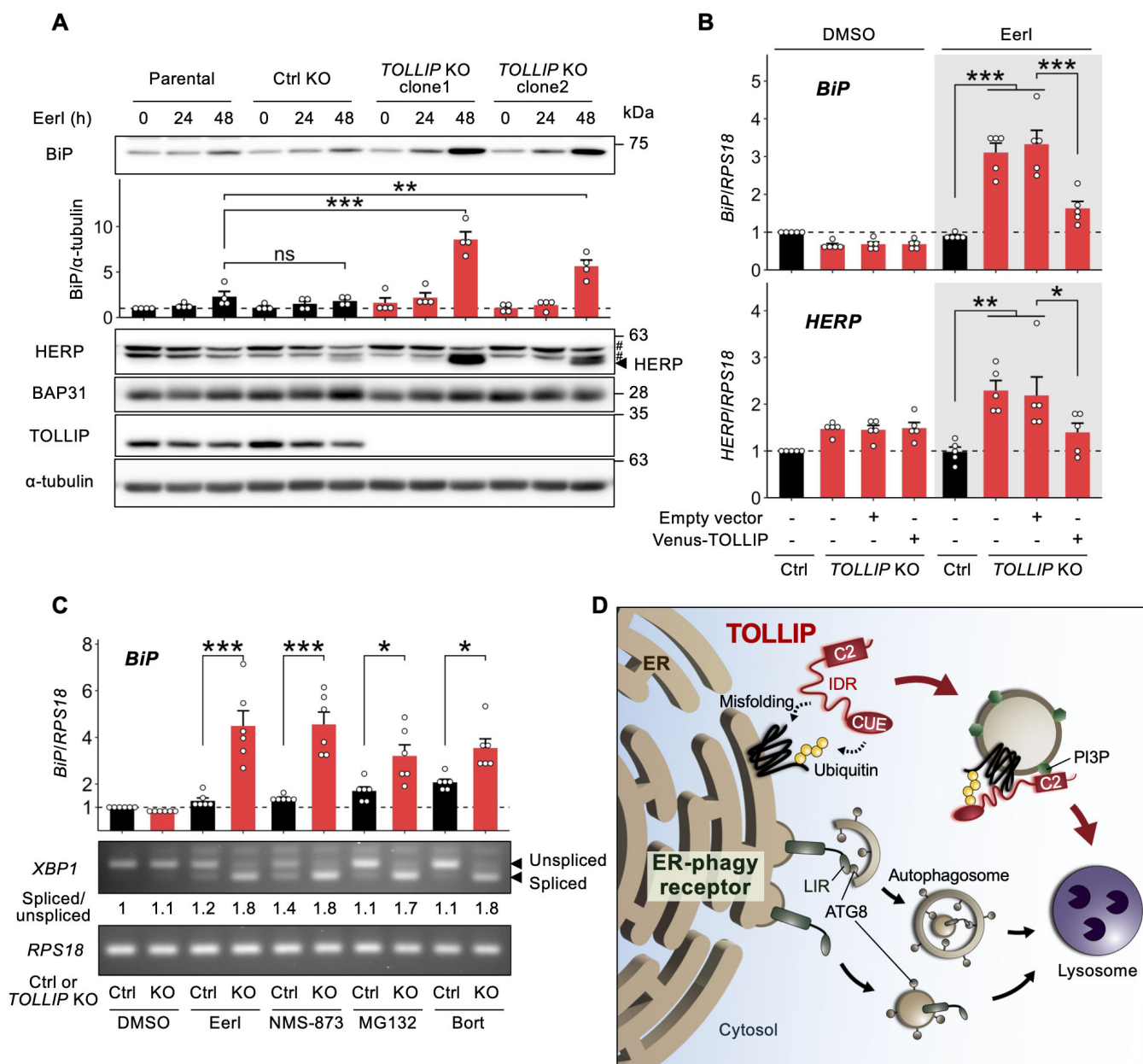


Figure 8. TOLLIP suppresses ER stress after ERAD inhibition.

A Immunoblot of parental, nontargeting sgRNA-introduced (Ctrl KO), or TOLLIP-KO HEK293A cells treated with 5 μ M eeyarestatin I (Eerl) for indicated times. The graph represents mean $\pm$ SEM of the BiP level normalized to α -tubulin ($n = 4$ independent experiments). #, unspecific bands.

B Quantitative PCR of ER stress markers. Ctrl or TOLLIP-KO (clone 1) HEK293A cells stably rescued with indicated constructs were treated with 5 μ M Eerl for 48 h. Data are represented as mean $\pm$ SEM of ER stress marker levels normalized to RPS18 ($n = 5$ independent experiments).

C Quantitative PCR of BiP (top) and semi-quantitative PCR of XBP1 (bottom). Ctrl or TOLLIP-KO (clone 1) HEK293A cells were treated with either 5 μ M Eerl, 1.5 μ M NMS-873, 0.8 μ M MG132, or 80 nM Bort for 20 h. The graph represents mean $\pm$ SEM of the BiP level normalized to RPS18, and numerical data represent means of the relative XBP1 mRNA splicing (spliced:unspliced) ($n = 6$ independent experiments).

D Model of TOLLIP-dependent recognition and lysosomal degradation of aberrant membrane proteins in the ER. In contrast to ER-phagy receptors, TOLLIP connects specific aberrant membrane proteins and PI3P-dependent lysosomal trafficking machinery without promoting bulk ER turnover.

Data information: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by one-way ANOVA with Dunnett's (A) or Tukey's (B) multiple comparison test or unpaired two-tailed Student's t test (C). Source data are available online for this figure.

P56S VAPB bearing cytosolic folding defects, the TOLLIP IDR recognizes Seipin harboring N88S and S90L mutations within the ER luminal consensus sequence for N-glycosylation, a post-translational

modification important for proper folding (Windpassinger *et al*, 2004). Missense mutations of membrane proteins sometimes cause severe folding defects not only in the neighboring regions of amino

acid substitutions but also in distal domains (Coelho *et al*, 2019; Marinko *et al*, 2019; Pobre-Piza *et al*, 2022). The Seipin mutants have been reported to form inclusion bodies in the ER (Ito *et al*, 2012; Kuijpers *et al*, 2013), indicating that their tertiary structure is drastically altered, which may be detectable at the cytosolic face of the ER by the TOLLIP IDR. Previously, this region of TOLLIP had been mapped to the binding region of various signaling molecules, including IRAK (Burns *et al*, 2000), TLR4 (Zhang & Ghosh, 2002), and STING (Pokatayev *et al*, 2020). The TOLLIP IDR may recognize partially unfolded or misfolded conformation of these native proteins. Future structural analysis of the IDR engaged with misfolded and native proteins is needed to determine how this region selectively recognizes diverse clients.

In addition to the IDR, we found that the ubiquitin-binding CUE domain is also required for the recognition and lysosomal degradation of aberrant membrane proteins. Our data, together with the recently reported RNF185-dependent clearance of Ebolavirus GP_{1,2}, suggest that ubiquitination serves as an important degradation signal in the lysosomal degradation of aberrant membrane proteins (Zhang *et al*, 2022a). The TOLLIP CUE domain has been reported to recognize three major types of ubiquitin modifications, namely, K48-linked, K63-linked, and monoubiquitin (Shih *et al*, 2003; Lu *et al*, 2014). Whether specific types of ubiquitin linkages are preferentially utilized during aberrant membrane protein turnover awaits future investigation. Notably, a CUE mutant still interacted with unmodified A53T-HP (Fig 3D) and tended to promote A53T-HP turnover, albeit less efficiently than WT TOLLIP (Fig EV1E and F), suggesting that substrate ubiquitination may play a degradation-enhancing, but not critical, role.

ERAD is the best-characterized proteolytic pathway for aberrant membrane proteins in the ER. In contrast, quality control factors that comprise lysosomal degradation pathways have remained poorly defined. Our present study, together with the recent literature, identified several cargo-specific factors, namely, DNAJB12 (He *et al*, 2021; Kennedy *et al*, 2022), RNF185 (Zhang *et al*, 2022a), and TOLLIP, which recognize and promote lysosomal degradation of aberrant membrane proteins. One of the remaining questions is the functional and mechanistic differences between diverse proteolytic pathways in the ER (Fig EV5E). Previous literature has suggested that aggregated ERAD-resistant membrane proteins are targeted to lysosomes (Lu *et al*, 2003; Kaushal, 2006; Fujita *et al*, 2007). Consistently, the clients of TOLLIP include mutants of VAPB and Seipin, both of which form visible aggregates within cells. There is also diversity within ER-to-lysosome delivery routes for aberrant membrane proteins. In addition to the TOLLIP-dependent pathway, which does not involve bulk ER turnover, ER-phagy also clears aberrant membrane proteins as evidenced by FAM134B-dependent I1061T NPC1 clearance (Schultz *et al*, 2018) and CCPG1-dependent A53T-HP clearance. In our study, A53T-HP may not have been sorted for selective degradation through ER-phagy, since bulk ER was also degraded by CCPG1. However, potential cargo selectivity in these ER-phagy receptor-dependent pathways cannot be ruled out, since both FAM134B and CCPG1 have been reported to recognize specific cargos in the cases of lysosomal degradation of secretory proteins (Fregno *et al*, 2018; Forrester *et al*, 2019; Ishii *et al*, 2023).

In addition to identifying several specific cargos cleared by TOLLIP, we also revealed that TOLLIP suppressed ER stress after

ERAD inhibition. Although the underlying mechanism remains to be explored, this raises the possibility that TOLLIP may clear endogenous ER stress-evoking membrane proteins that accumulate when ERAD is blocked. Consistent with our assumption, a very recent paper reported that TOLLIP depletion leads to the accumulation of endogenous ER-localized membrane proteins TMEM63A and Derlin-1 (Zhang *et al*, 2022b). Inhibition of ERAD and the subsequent ER stress have been implicated in various neurodegenerative diseases, including Huntington's disease (Duennwald & Lindquist, 2008), ALS (Nishitoh *et al*, 2008; Fujisawa *et al*, 2012; Homma *et al*, 2013), and Alzheimer's disease (Abisambra *et al*, 2013). Because TOLLIP is highly expressed in the brain (Burns *et al*, 2000), our finding showing that TOLLIP suppresses ER stress may be relevant in the context of these diseases. Considering all of our findings together, bolstering TOLLIP function emerges as a therapeutic strategy for diverse diseases that involve the accumulation of toxic misfolded membrane proteins and/or ER stress evoked by disrupted ERAD.

Materials and Methods

A complete list of cell lines, plasmids, antibodies, oligonucleotides, chemicals, software, and kits used in this study is reported in Appendix Table S1.

Cell lines

HEK293 cells, HEK293A cells, and NSC34 cells were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, D5796), HeLa cells were maintained in low-glucose DMEM (Wako, 041-29775), and SH-SY5Y cells were maintained in DMEM/Ham's F-12 (Sigma, D8062). Each culture medium was supplemented with 10% fetal bovine serum (FBS) (BioWest, S1560-500 or Gibco, 10270-106). All but DMEM/Ham's F-12 were also supplemented with 100 units/ml penicillin G (Meiji Seika, 01028-85). All cell lines were grown in a 5% CO₂ incubator at 37°C and confirmed to be negative for mycoplasma. For inducing gene expression in tetracycline-inducible cell lines, cells were treated with 0.5 µg/ml tetracycline (Sigma-Aldrich, T7660) for at least 24 h.

Plasmids

cDNAs encoding human α -synuclein (NM_000345), p62 (NM_003900), OPTN (NM_001008211), NBR1 (NM_031862), TOLLIP (NM_019009 with c.417G > A), LAMP1 (NM_005561 with c.556C > A), NEDD4 (NM_006154 with c.779G > A and c.836A > G), SAR1A (NM_020150), CD147 (NM_198589 with c.195C > T and c.234C > G), CCPG1 (NM_020739 with c.940T > C, c.1126T > C, and c.1252T > C), E-Syt1 (NM_015292), VAPB (NM_004738 with c.732G > A), Seipin (NM_032667), α 1-antitrypsin (NM_001127701), and TTR (NM_000371), and mouse Tollip (NM_023764) were amplified by PCR and inserted into pcDNA3 (Invitrogen), pcDNA4/TO (Invitrogen), or pTRE-Tight-BI-DsRed-Express (Clontech). Truncated or mutated constructs were prepared by PCR-mediated site-directed mutagenesis. A53T-HP was generated by inserting A53T α -synuclein, the hairpin region (1–97 aa) of E-Syt1 as determined in a previous report (Giordano *et al*, 2013), and an HA or a FLAG epitope into pcDNA4/TO. 2xFYVE probe was generated by inserting two copies of the FYVE

domain (147–223 aa) of mouse Hgs (NM_001159328) and fluorescent proteins into pcDNA3. VN-A53T-HP for BiFC assay was generated by inserting mVenus (1–155 aa) and A53T-HP into pcDNA4/TO, and VC-TOLLIP was generated by inserting mVenus (156–239 aa) and TOLLIP into pcDNA3. ss-mCherry-EGFP-KDEL was generated by inserting the signal sequence (1–19 aa) of human BiP (NM_005347), mCherry, EGFP, and the KDEL sequence into pcDNA3. Single-guide RNAs (sgRNAs) targeting *TOLLIP* (5'-CTACAGACAGCGGCATCCC-3'), *CCPG1* (5'-CGTCGTCTAAAGGCAGGACT-3'), *FAM134B* (5'-ACTCTTTGGCAGCAACCGTG-3'), *RTN3* (5'-GCGCGCCTTACCCGCACAGG-3'), *SEC62* (5'-ATCATTTGCTCATAACTGG-3'), *TEX264* (5'-GCCACAGTGACGTTGCGGAT-3'), *FIP200* (5'-AGATCGAGCTCGTTTGCTTG-3'), and *ATG7* (5'-CTAGGACGTTGATGGTAAGT-3') were designed using CHOPCHOP (<https://chopchop.cbu.uib.no>). These sgRNA sequences and nontargeting control sequence (5'-GCGCGAGCGACGAAACGACA-3') were inserted into pSpCas9(BB)-2A-Puro V2.0 (a gift from Dr. F. Zhang) (Addgene, 62988).

Transfection

Plasmid transfection was performed using Polyethylenimine “MAX” (Polysciences, 24765) or Lipofectamine 3000 (Invitrogen, L3000008). Cells were transfected at 90% confluency, according to the manufacturer's instructions. In most experiments, cells were harvested or analyzed 48 h after plasmid transfection. siRNA-mediated knock-down was performed using Lipofectamine RNAiMAX (Invitrogen, 13778500) and Opti-MEM (Invitrogen, 31985) at a final siRNA concentration of 15 or 20 nM by reverse transfection, according to the manufacturer's instructions. Plasmid transfection into siRNA-treated cells was performed 36 h after siRNA transfection. For the immunoblot analysis of mCherry processing after TOLLIP overexpression, after 8–10 h of plasmid transfection, cells were suspended, diluted twofold with fresh medium, and seeded again into new plates, so cells would not reach over-confluence.

Generation of knockout cell lines

For CRISPR/Cas9-mediated knockout, cells were transfected with pX459 encoding sgRNAs. Forty-eight hours after transfection, the cells were selected by 2 µg/ml puromycin (Gibco, A11138-03) treatment for another 48 h. The selection medium was replaced with fresh standard medium, and the cells were grown for 4 days. Limiting dilution was performed to obtain single-cell clones. The knockout status of the clones was confirmed by sequencing genomic DNA (Eurofins Genomics) and/or immunoblotting.

Generation of stable cell lines

HEK293 cells stably expressing Tet repressor (Nagai et al, 2009) were transfected with pcDNA4/TO encoding A53T-HA-HP and selected with 5 µg/ml blasticidin (Gibco, A1113903) and 400 µg/ml zeocin (Gibco, R25001). HEK293A cells were transfected with pcDNA6/TR and pcDNA4/TO encoding either mCherry-A53T-HA-HP, mCherry-HA-VAPB, or mCherry-HA-P56S VAPB, and selected with 5 µg/ml Blasticidin and 300 or 400 µg/ml Zeocin. A single clone of cells expressing mCherry-A53T-HA-HP was obtained by limiting dilution. *TOLLIP*-KO HEK293A cells (clone 1) were transfected with intact pcDNA3/GW or the same plasmid encoding

Venus-TOLLIP and were subsequently selected with 500 µg/ml geneticin (Gibco, 10131035).

Cell lysis and immunoblotting

Cells were lysed in 2× sample buffer (80 mM Tris-HCl pH 8.8, 80 µg/ml bromophenol blue, 28.8% glycerol, and 4% SDS) supplemented with 10 mM DTT and protease inhibitors (1 mM PMSF and 5 µg/ml leupeptin). The lysates were incubated at 65°C for 10 min, briefly sonicated with an ultrasonic homogenizer (SMT, UH-50), and incubated again at 65°C for 5 min. SDS-insoluble debris was cleared by centrifugation at 17,700 g for 5 min. In Figs 1E, 4A, 7A, 8A, EV3B and G, EV5B and C, and Appendix Fig S1B, cells were lysed in TNE buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, and 10 mM EDTA) supplemented with 1% SDS and protease inhibitors (1 mM PMSF, 5 µg/ml leupeptin, and 20 mM NEM). The lysates were incubated at 65°C for 10 min, briefly sonicated, and centrifuged at 17,700 g for 5 min. Quantification of proteins in the supernatants was performed with DC protein assay (Bio-Rad, 5000116), and the supernatants were adjusted to equal protein concentrations, which were mixed with an equal volume of 2× sample buffer supplemented with 20 mM DTT.

The samples were then resolved by SDS-PAGE and transferred onto Immobilon-P PVDF membranes (Millipore, IPVH00010). Where indicated, the membranes were stained with Ponceau S (APRO Science, SP-4030) before blocking, according to the manufacturer's instructions. Blocking was performed with 5% skim milk (Megmilk Snow Brand) in TBS-T. After incubating the membranes with the indicated primary antibodies and corresponding HRP-linked secondary antibodies, the chemiluminescent signals enhanced by ECL Select (Cytiva, RPN2235) were detected using Fusion Solo 7S (M&S Instruments). Adjustment of contrast and brightness, and quantification were performed using Fiji software (Schindelin et al, 2012).

Differential centrifugation

Cells were rinsed with ice-cold PBS and suspended in hypotonic extraction buffer (10 mM HEPES-KOH pH 7.5, 25 mM KCl, and 1 mM EGTA) supplemented with protease inhibitors (1 mM PMSF and 5 µg/ml leupeptin). After 25 strokes with a Potter-Elvehjem homogenizer, sucrose was added to the homogenates at a final concentration of 250 mM. The homogenates were centrifuged at 700 g for 5 min. The pellets were washed twice with PBS to obtain the P0.7 (nuclear) fraction, and then, the supernatants were centrifuged again to obtain postnuclear supernatants. These supernatants were then centrifuged at 7,000 g for 10 min. The pellets were washed twice with PBS to obtain the P7 (mitochondrial) fraction, while the supernatants were centrifuged again to obtain postmitochondrial supernatants. The supernatants were then ultracentrifuged at 100,000 g for 60 min (HITACHI, CS100GX). The supernatants (S100, cytosol) were collected, and the pellets were washed twice with PBS to obtain the P100 (microsomal) fraction. The pellet fractions were lysed in hypotonic extraction buffer supplemented with 1% Triton X-100, 250 mM sucrose, and the protease inhibitors, briefly sonicated, and centrifuged at 17,700 g for 5 min to remove debris. The S100 fraction was supplemented with 1% Triton X-100. Protein quantification was performed by DC protein assay, and the

lysates adjusted to equal protein concentrations were mixed with an equal volume of 2× sample buffer supplemented with 20 mM DTT. The samples were denatured at 65°C for 10 min. When only the P100 and S100 fractions were analyzed, the centrifugation steps performed at 7,000 g were omitted.

Detergent solubility assay

Cells were lysed in TNE buffer supplemented with 1% Triton X-100 and protease inhibitors (1 mM PMSF and 5 µg/ml leupeptin). After centrifugation at 17,700 g for 10 min at 4°C, the supernatants were mixed with an equal volume of 2× sample buffer supplemented with 20 mM DTT and denatured at 65°C for 10 min (TX-100 soluble fraction). The pellets were washed with TNE buffer and lysed in TNE buffer supplemented with 2% SDS and 10 mM DTT. The lysates were incubated at 65°C for 10 min, briefly sonicated, and centrifuged at 17,700 g for 5 min. The supernatants were mixed with an equal volume of 2× sample buffer supplemented with 10 mM DTT and denatured at 65°C for 10 min (TX-100 insoluble fraction). To compare the relative amount of proteins of interest between the TX-100 soluble and insoluble fractions, an equal volume of TNE buffer was used for cell lysis and pellet lysis.

Coimmunoprecipitation

Cells were lysed in TNE buffer supplemented with 1% Triton X-100 and protease inhibitors (1 mM PMSF, 5 µg/ml leupeptin, and 20 mM NEM). After centrifugation at 17,700 g for 10 min at 4°C, the supernatants were subjected to immunoprecipitation. For IP of HA-tagged proteins, the supernatants were first incubated with anti-HA antibody (Roche, 11867431001) or IgG1 isotype control (Bio X Cell, BE0290) for 4–6 h, subsequently added with Protein G Sepharose (Cytiva, 17061802), and incubated again for 45 min. The beads were washed twice with buffer 1 (20 mM Tris–HCl pH 7.5, 500 mM NaCl, 5 mM EGTA, and 1% Triton X-100) and once with buffer 2 (20 mM Tris–HCl pH 7.5, 150 mM NaCl, and 5 mM EGTA). The proteins were eluted with 2× sample buffer supplemented with 20 mM DTT at 65°C for 10 min. For IP of FLAG-tagged proteins, anti-DYKDDDDK tag antibody beads (FUJIFILM Wako, 016-22784) were mixed with the supernatants for 60 min, washed, and eluted with 2× sample buffer. Alternatively, for IP of FLAG-tagged proteins in the microsomal fraction, anti-FLAG M2-agarose gel (Sigma-Aldrich, A2220) was mixed with the lysates for 60 min, washed, and eluted with 3xFLAG peptide (Sigma-Aldrich, F4799) dissolved in buffer 2 at 15°C for 50 min. The eluates were mixed with an equal volume of 2× sample buffer supplemented with 20 mM DTT and denatured at 65°C for 10 min.

In sequential IP, FLAG-tagged proteins in lysates were first immunoprecipitated with anti-FLAG M2-agarose gel and eluted with 3xFLAG peptide (M&S TechnoSystems, GEN-3-FLAG-5) dissolved in buffer 2 supplemented with protease inhibitors (1 mM PMSF, 5 µg/ml leupeptin, and 20 mM NEM) at 15°C for 45 min. The eluates were then diluted 20-fold with RIPA buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) supplemented with the protease inhibitors, and HA-tagged proteins were immunoprecipitated as described above.

Substrate ubiquitination analysis

Cells were lysed in TNE buffer supplemented with 1% SDS and protease inhibitors (1 mM PMSF, 5 µg/ml leupeptin, and 20 mM NEM). The lysates were incubated at 65°C for 10 min, briefly sonicated, and centrifuged at 17,700 g for 5 min. The supernatants were diluted tenfold with TNE buffer supplemented with 1% Triton X-100 and the protease inhibitors and then subjected to IP as described above. To detect ubiquitinated species via immunoblot analysis, PVDF membranes were blocked with 5% BSA (Iwai Chemicals, A001).

Promoter shut-off pulse-chase assay

A T-Rex system (Invitrogen)-based pulse-chase assay for measuring the turnover of A53T-HP was performed based on a reported protocol with modifications (Scotter *et al*, 2014). Cells were cotransfected with pcDNA4/TO/mCherry-A53T-HA-HP, pcDNA6/TR, and pcDNA3/GW/FLAG-TOLLIP variants. The amount of pcDNA6/TR was at least 15-fold higher than that of pcDNA4/TO to prevent leaky expression of mCherry-A53T-HA-HP in the absence of tetracycline. After 8–10 h, the cells were seeded into plates coated with Cellmatrix Type I-C (Nitta Gelatin, KP-4100) in medium containing 0.5 µg/ml tetracycline to induce the expression of mCherry-A53T-HA-HP. After 24 h in culture, the cells were rinsed with PBS, and the medium was replaced with medium without tetracycline. The cells were incubated for another 24 h to allow for the clearance of mCherry-A53T-HA-HP transcripts. Twenty-four hours after the tetracycline washout was set to time 0, and the cells were harvested for immunoblot and quantitative PCR analyses at the indicated time points. For the immunoblot analysis, the linearity of signals was confirmed by preparing a dilution series of one of the samples.

The turnover of VAPB and Seipin variants was measured using Tet-Off system (Clontech), since their degradation was not clearly detected by the T-Rex system-based assay due to the relatively slow clearance of exogenous VAPB and Seipin transcripts. Cells were cotransfected with pTRE-Tight-BI-DsRed-Express/mCherry-HA-VAPB variants or pTRE-Tight-BI-DsRed-Express/Seipin-HA-mCherry variants and pTet-Off. After 24 h, the cells were treated with 2.5 µg/ml tetracycline to halt the transcription of VAPB and Seipin variants. Twenty-four hours after the tetracycline addition was set to time 0, and the cells were harvested for immunoblot and quantitative PCR analyses at the indicated time points.

Quantitative PCR analysis

Total RNA was isolated from cells using Isogen (Nippongene, 319-90211) and reverse transcribed with ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, FSQ-301), according to the manufacturer's instructions. Quantitative PCR was prepared using either FastStart Essential DNA Green Master (Roche, 06924204001) or KAPA SYBR Fast qPCR Kit (Kapa Biosystems, KK4602), cDNA product, and the transcript-specific forward and reverse primers. Quantitative PCR was then performed on LightCycler 96 (Roche) or QuantStudio 1 (Thermo Fisher Scientific), and the data were normalized to that of *RPS18*.

Semi-quantitative PCR for *XBP1* mRNA splicing

Total RNA was reverse transcribed as described above and amplified in reactions containing cDNA product, forward and reverse primers for *XBP1*, dNTP mix (Promega, U1515), 5× Green Go Taq Reaction Buffer (Promega, M7911), and Taq polymerase (prepared in house). Amplified fragments covering a 26-nucleotide intron and flanking exon sequences were separated on 3% agarose gels, visualized by EtBr staining, and detected using UVP GelSolo, M-20V (Analytik Jena).

Protein purification

The TOLLIP IDR or CUE domain and HSP70 encoded in pGEX-6P-1 (Cytiva) were expressed in *Escherichia coli* BL21 cells. Expression was induced in LB medium supplemented with 0.3 mM IPTG at 25°C for 5 h, and then, the cells were pelleted and frozen overnight at −80°C. Thawed cells were suspended in PBS supplemented with 10 mM EDTA and 1% Triton X-100, homogenized, and centrifuged at 26,800 g for 30 min. The supernatants were incubated with glutathione Sepharose 4B (Cytiva, 17075601) at 4°C for 3 h. The beads were then washed six times with PBS supplemented with 10 mM EDTA and eluted with 10 mM reduced L-glutathione (Sigma-Aldrich, G4251) in 50 mM Tris-HCl pH 8.0 using Poly-Prep Chromatography Columns (Bio-Rad). The eluates were poured into cellulose tubes (Eidia, UC18-32-100 18/32) and dialyzed in PBS overnight at 4°C to remove glutathione. The purity and concentration of the final products were estimated by Coomassie Blue staining of SDS-PAGE gels.

In vitro binding assay

An *in vitro* binding assay was performed based on a reported protocol with modifications (Hjerpe et al, 2016). Purified GST-tagged proteins (1–5 μM) were incubated with 45 or 90 nM Luciferase (Promega, E1701) in a buffer (25 mM HEPES-Tris pH 7.4, 50 mM KCl, and 5 mM MgCl₂) supplemented with 2 mM DTT at 4 or 42°C for 60–120 min. The reactions were then diluted at least fourfold with TNE buffer, supplemented with glutathione Sepharose (Cytiva, 17075601), and incubated at 4°C for 30–150 min. The beads were washed three times with TNE buffer and eluted with 15 mM reduced L-glutathione in 50 mM Tris-HCl pH 8.0 at 15°C for 35 min. The eluates were mixed with an equal volume of 2× sample buffer supplemented with 20 mM DTT and denatured at 65°C for 10 min.

Fluorescence microscopy

For fluorescence microscopy of fixed cells, cells grown on coverslips were rinsed with PBS, fixed with 4% paraformaldehyde in PBS for 10 min, permeabilized with 0.2% Triton X-100 in PBS, blocked with 5% BSA in TBS-T for 3 h, and incubated with the indicated primary antibodies overnight at 4°C. After washing three times with PBS, the cells were incubated with fluorophore-conjugated secondary antibodies at room temperature for 90 min, subjected to nuclear staining with Hoechst 33342 (Dojindo, 346-07951) for 5 min, and mounted with Fluoromount (Diagnostic BioSystems, K024). The samples were observed with TCS SP5 confocal microscope (Leica

Microsystems) through a 63×/1.40 oil objective. For live cell imaging, cells grown on glass bottom dishes were subjected to lysosome staining with LysoTracker Red DND-99 (Invitrogen, L7528) for 2 h and observed similarly without fixation.

A colocalization analysis and quantification of punctate structures were performed with Fiji software. Briefly, colocalization analysis between two channels was performed with the Coloc 2 plugin to calculate Pearson's R values in manually determined ROIs. The accumulation of mCherry-EGFP-A53T-HP in lysosomal acidic puncta was quantified by first detecting mCherry puncta using the Analyze Particles function, defining acidic puncta as puncta with an intensity ratio of mCherry:EGFP higher than 1.25, and quantifying mCherry intensity in acidic puncta relative to the total mCherry intensity in each cell. The accumulation of mCherry-A53T-HP in PI3P puncta was quantified as follows: First, PI3P puncta were detected in the Venus-2xYFVE channel using the Analyze Particles function. Only cells with at least eight detected PI3P puncta were further analyzed. The average number of detected PI3P puncta was 38 (siCtrl) and 33 (siTOLLIP) in Fig 4B, and 39 (CFP), 36 (WT), 37 (C2^{mut}), 48 (IDR^{mut}), and 37 (ΔCUE) in Fig 4C. PI3P puncta containing more than 0.15% of the total mCherry-A53T-HP signal in each cell were defined as mCherry-positive PI3P puncta. The accumulation of mCherry-A53T-HP in LAMP1 puncta was quantified similarly: cells with at least seven detected LAMP1 puncta were analyzed, and LAMP1 puncta containing more than 0.2% of the total mCherry-A53T-HP signal were defined as mCherry-positive LAMP1 puncta. The average number of detected LAMP1 puncta was 50 (siCtrl), 33 (siTOLLIP), 32 (siTOLLIP + WT), 45 (siTOLLIP + LIR^{mut}), 28 (siTOLLIP + C2^{mut} #1), 38 (siTOLLIP + IDR^{mut}), and 38 (siTOLLIP + ΔCUE) in Fig 2G, and 37 (siCtrl), 21 (siCtrl + TOLLIP), 34 (siVPS34), 31 (siVPS34 + TOLLIP), 28 (SAR405), and 18 (SAR405 + TOLLIP) in Fig EV3A. To prepare representative images, contrast and brightness were adjusted using Fiji software, and cropping was performed using Adobe Illustrator.

Correlative light and electron microscopy

Cells grown on glass bottom dishes with grids (MatTek, P35G-1.5-14-C-GRID) were fixed with 4% paraformaldehyde in 0.1 M PB for 30 min and rinsed with 0.1 M PB, and fluorescence and bright-field images were obtained with a BZ-X810 microscope (Keyence) through a 60× oil objective. After imaging, the cells were fixed with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M PB for 1 h at room temperature, followed by 1% osmium tetroxide and 1.5% potassium ferrocyanide in 0.1 M cacodylate buffer pH 7.4 for 1 h on ice and rinsed with MilliQ; then, the coverslips were removed from the dishes by Coverslip Removal Fluid (MatTek, P DCF OS 30). The coverslips were dehydrated through series of increasing ethanol concentrations (70, 80, 90, 95, and 100%), infiltrated with QY-1, embedded in Durcupan ACM, and polymerized at 60°C for 2 days. A 1 × 1 mm area containing the cells of interest was cut out, and serial ultrathin sections (approximately 50 nm thick) were prepared with a diamond knife and collected on Formvar-coated grids. The sections were stained with 4% uranyl acetate for 5 min and with Reynolds' lead citrate for 2 min, and extensively washed with MilliQ. Electron microscopic images were acquired using a transmission electron microscopy JEM-1200EX (JEOL) equipped with a Veleta 2 × 2 k side-mounted CCD camera.

Disorder prediction

The amino acid sequence of human TOLLIP was obtained from the NCBI CCDS database (<https://www.ncbi.nlm.nih.gov/projects/CCDS>). The disordered regions were predicted using DISOPRED3 (<http://bioinf.cs.ucl.ac.uk/psipred>), PONDR (<http://www.pondr.com>), and SPOT-Disorder2 (<https://sparks-lab.org/server/spot-disorder2>).

Quantification and statistical analysis

All statistical data were calculated using RStudio. Comparisons of two groups of data were performed by unpaired two-tailed Student's *t* test or one-sided Wilcoxon rank sum test, and multiple comparison tests were performed by one-way analysis of variance (ANOVA) followed by Dunnett's or Tukey's multiple comparison test. The *P* values < 0.05 were considered to be significant. "ns" in the graphs denotes differences that were not significant. The detailed statistical information of each experiment, including the numbers of independent experiments performed on different days (*n*), is shown in the figures and corresponding legends.

Data availability

This study includes no data deposited in external repositories.

Expanded View for this article is available [online](#).

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Author contributions

Yuki Hayashi: Conceptualization; formal analysis; funding acquisition; investigation; visualization; methodology; writing – original draft; writing – review and editing. **Sho Takatori:** Investigation; methodology; writing – review and editing. **Waleed Y Warsame:** Investigation. **Taisuke Tomita:** Supervision; funding acquisition; writing – review and editing. **Takao Fujisawa:** Supervision; funding acquisition; writing – review and editing. **Hidehiko Ichijo:** Supervision; funding acquisition; writing – review and editing.

Disclosure and competing interests statement

The authors declare that they have no conflict of interest.

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Expanded View Figures

Figure EV1. The TOLLIP C2, IDR, and CUE domains are required for degradation of an aberrant ER membrane protein.

- A Lysosomal degradation of A53T-HP in HEK293A cells transfected with indicated siRNAs and mCherry-A53T-HP. The graph represents mean $\pm$ SEM of mCherry processing ($n = 4$ independent experiments).
- B As in (A), but HeLa cells were used.
- C Immunofluorescence of HEK293A cells transfected with mCherry-EGFP-A53T-HP and stained with an anti-RAB7 antibody. Scale bars, 10 and 2 μ m (insets) ($n = 3$ independent experiments).
- D The effect of TOLLIP overexpression on lysosomal degradation of cytosolic A53T α -synuclein, ER membrane protein E-Syt1, and A53T-HP. HEK293A cells were transfected with indicated mCherry-tagged constructs and FLAG-TOLLIP. Numerical data represent mean $\pm$ SEM of TOLLIP-dependent fold change in mCherry processing for each mCherry-tagged construct ($n = 3$ independent experiments).
- E, F Turnover of A53T-HP monitored by promoter shut-off assay. HEK293A cells were transfected with HA-tagged mCherry-A53T-HP harboring a tetracycline (Tet)-responsive promoter, Tet repressor, and FLAG-TOLLIP variants. Cells were treated with 0.5 μ g/ml Tet for 24 h to induce the expression of mCherry-A53T-HP and then replenished with fresh medium without Tet to allow for RNA decay. Twenty-four hours after Tet washout was set to time 0, and the remaining mCherry-A53T-HP protein expression level was evaluated at indicated time points. The graphs represent mean $\pm$ SEM of the turnover of mCherry-A53T-HP protein quantified by immunoblotting and RNA quantified by real-time quantitative PCR in parallel experiments to confirm comparable clearance of the transcripts in all conditions ($n = 3$ independent experiments). Statistical significance was determined at the final time points. Δ TBD, Δ 1–44; C2^{mut} #1, R123A; IDR^{mut}, 181–229 > (GGGGG)₉GGGG; Δ IDR, Δ 181–229; CUE^{mut}, L267A and L268A; LIR^{mut}, W133A, T134A, H135A, I136A, W151A, Y152A, S153A, and L154A.

Data information: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by one-way ANOVA with Dunnett's multiple comparison test (A, B, E and F).

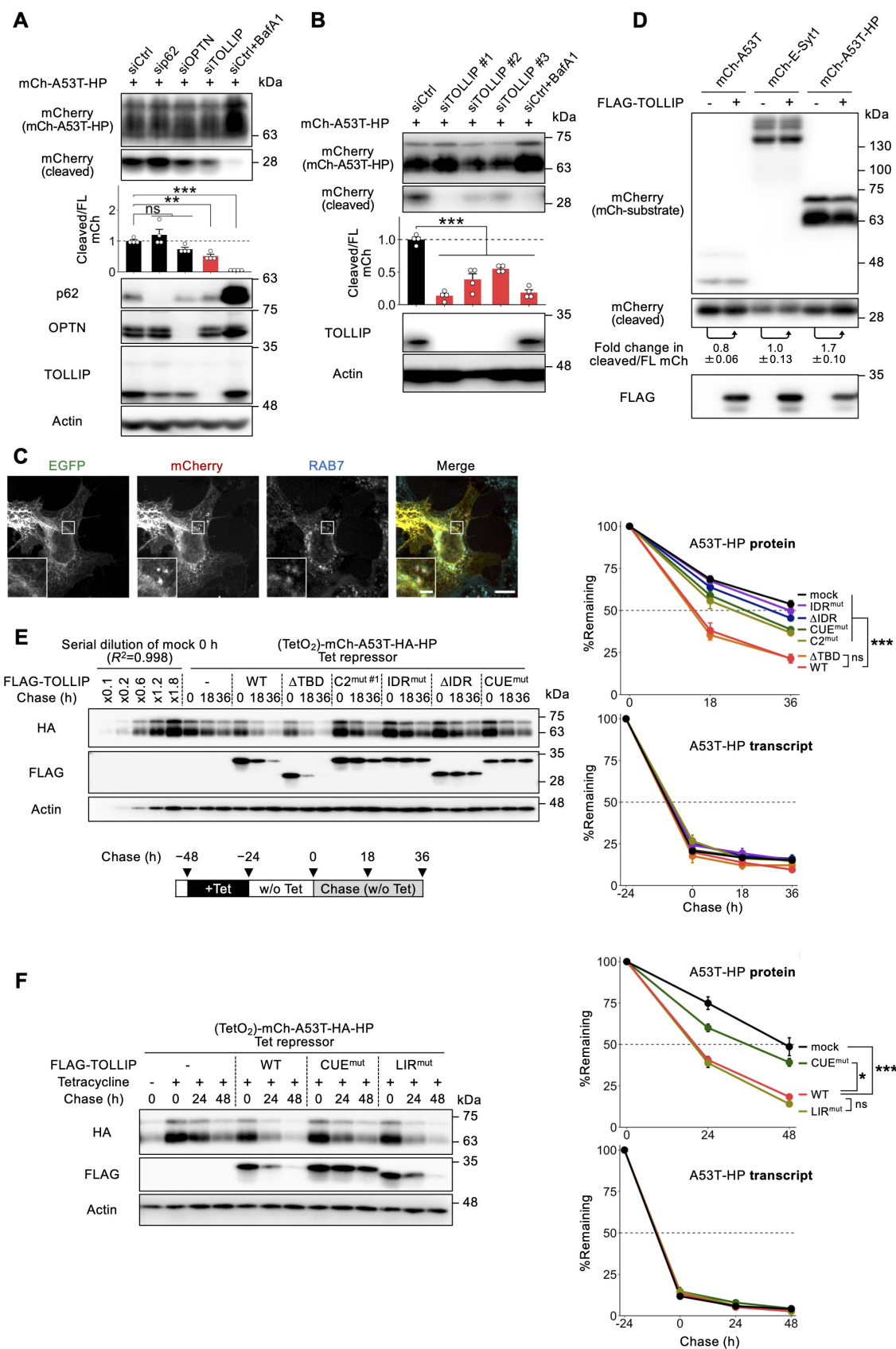


Figure EV1.

Figure EV2. Ubiquitination enhances lysosomal degradation of an aberrant ER membrane protein.

- A Co-IP of FLAG-TOLLIP variants with endogenous ubiquitin conjugates. HEK293A cells were transfected with FLAG-TOLLIP variants and lysed in buffers containing 1% Triton X-100 (TX-100) or 0.1% SDS (RIPA). Co-IP was performed with an anti-FLAG antibody. Since ubiquitin conjugates coimmunoprecipitated from RIPA lysates can be regarded as those covalently attached to TOLLIP, the differences in the amount of ubiquitin conjugates in the TX-100 lysed and RIPA lysed fractions were regarded as ubiquitinated endogenous proteins complexed with TOLLIP. The graph represents mean $\pm$ SEM of these differences ($n = 3$ independent experiments).
- B *In vitro* binding assay of HSP70 and heat-denatured luciferase. Recombinant GST fusion proteins were incubated with recombinant luciferase at 4 or 42°C for 120 min and purified using glutathione Sepharose. The data are representative of four experiments performed with the same batches of purified proteins.
- C Denaturing IP to evaluate the expression level of ubiquitinated A53T-HP. HEK293A cells were transfected with indicated siRNAs and HA-tagged mCherry-A53T-HP, treated with or without 100 nM BafA1 for 20 h, and lysed under denaturing conditions. The lysates were subjected to IP using an anti-HA antibody. The graph represents mean $\pm$ SEM of the BafA1-dependent fold increase in the amount of ubiquitinated species (top panel) normalized to unmodified species (second panel) ($n = 3$ independent experiments).
- D Lysosomal degradation of A53T-HP in HEK293A cells transfected with FLAG-NEDD4 WT or catalytically inactive C867S (CS) and mCherry-A53T-HP. The graph represents mean $\pm$ SEM of mCherry processing ($n = 3$ independent experiments). In parallel, the cells were lysed under either nondenaturing or denaturing conditions and subjected to IP with an anti-HA antibody to evaluate the interaction of A53T-HP with NEDD4 or the ubiquitination status of A53T-HP, respectively. Although the C867S mutation in the catalytic core diminishes NEDD4 E3 activity, the mutant retained substrate-binding capacity, possibly because distal WW domains recognized α -synuclein (Tofaris et al, 2011).

Data information: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by one-way ANOVA with Dunnett's multiple comparison test (C and D).

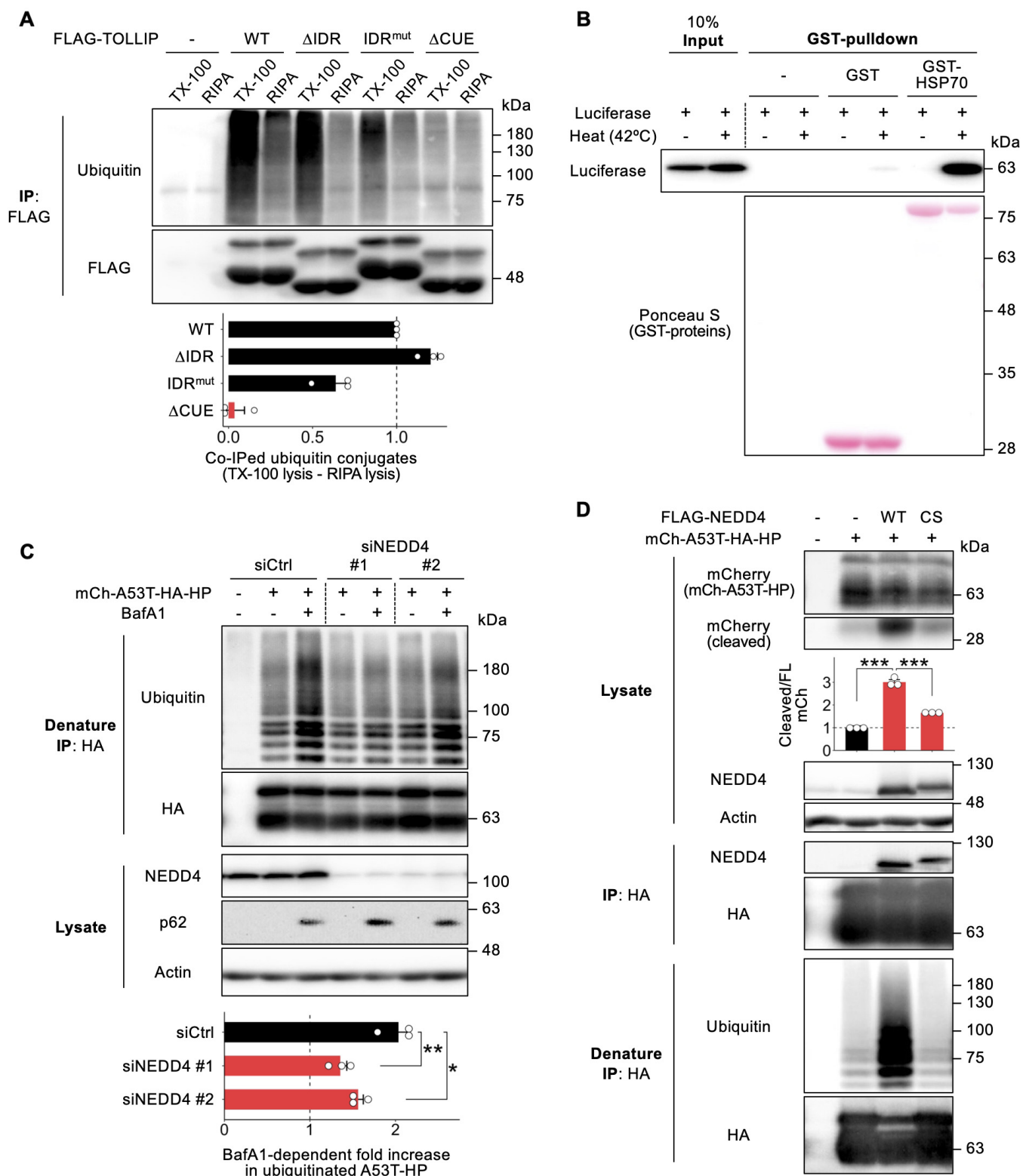


Figure EV2.

Figure EV3. TOLLIP promotes PI3P-dependent lysosomal trafficking of an aberrant ER membrane protein.

- A Fluorescence microscopy imaging to monitor the lysosomal delivery of A53T-HP. HEK293A cells were transfected with indicated siRNAs and constructs, treated with 100 nM BafA1 with or without 0.5 μ M SAR405 for 12 h, and stained with an anti-FLAG antibody. The percentage of LAMP1 puncta containing mCherry-A53T-HP in each cell was quantified. From 12 to 26 cells in each condition pooled from $n = 2$ independent experiments were analyzed. Scale bars, 10 and 2 μ m (insets).
- B Validation of the loss of VPS34 function in an experiment conducted in parallel with (A) except for BafA1 treatment ($n = 2$ independent experiments).
- C Another example of CLEM images as in Fig 4E. Serial 50 nm sections are shown. Black arrowhead indicates a single membrane surrounding a vesicle and white arrowheads indicate a smooth ER-like double-membrane structure located in close proximity to the vesicle. N, nucleus; M, mitochondria. Scale bars, 500 and 50 nm (inset).
- D Turnover of A53T-HP monitored by promoter shut-off assay in HEK293A cells transfected with indicated plasmids as in Fig EV1E and F. The graph represents mean $\pm$ SEM of the turnover of mCherry-A53T-HP protein ($n = 3$ independent experiments).
- E Fractionation into the postnuclear membrane (P100) and the cytosol (S100) of HEK293A cells transfected with Venus-TOLLIP and mCherry-A53T-HP and treated with or without 10 μ M SAR405 for 24 h. In parallel, whole-cell lysates (WCLs) were prepared to confirm TOLLIP- and PI3P-dependent lysosomal degradation of A53T-HP under these experimental conditions ($n = 3$ independent experiments).
- F Lysosomal degradation of A53T-HP in parental or *CCPG1*-KO HEK293A cells transfected with indicated siRNAs and mCherry-A53T-HP. The graph represents mean $\pm$ SEM of mCherry processing ($n = 6$ independent experiments).
- G Validation of macroautophagy-defective HEK293A cell lines. Cells with indicated genotypes were treated with or without the macroautophagy inducer 1 μ M rapamycin for 7 h and 200 nM BafA1 for 4 h. Impaired LC3-II flux (BafA1-dependent increase in the LC3-II level) and p62 accumulation suggest that macroautophagy is defective in *FIP200*-KO and *ATG7*-KO cells ($n = 2$ independent experiments).
- H Lysosomal degradation of A53T-HP in parental or *FIP200*-KO HEK293A cells transfected with indicated constructs. The graph represents mean $\pm$ SEM of mCherry processing ($n = 3$ independent experiments).
- I Turnover of A53T-HP monitored by promoter shut-off assay in HEK293A cells with indicated genotypes as in (D). The graph represents mean $\pm$ SEM of the turnover of mCherry-A53T-HP protein ($n = 3$ independent experiments).

Data information: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by one-way ANOVA with Dunnett's (D and I) or Tukey's (F) multiple comparison test or unpaired two-tailed Student's t test (A and H).

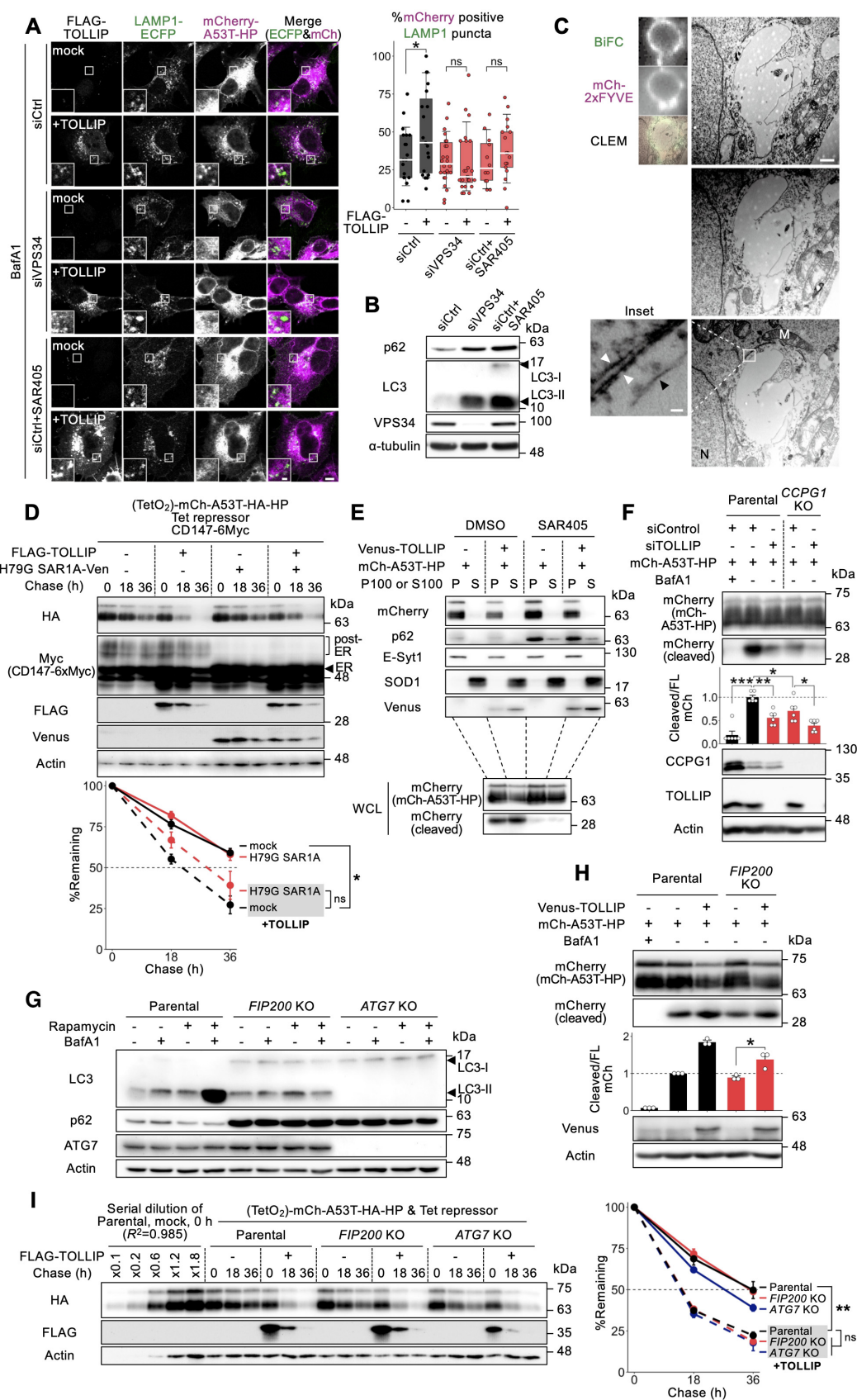


Figure EV3.

Figure EV4. TOLLIP clears disease-linked mutant membrane proteins.

- A Turnover of VAPB variants monitored by promoter shut-off assay in HEK293A cells transfected with indicated siRNAs and HA-tagged VAPB variants as in Fig 7B. The graphs represent mean $\pm$ SEM of the turnover of mCherry-HA-VAPB protein quantified by immunoblotting ($n = 3$ independent experiments) and RNA quantified by real-time quantitative PCR in parallel experiments ($n = 2$ independent experiments). Statistical significance was determined at the final time point.
- B, C Co-IP of HA-tagged P56S VAPB and N88S Seipin with Venus-TOLLIP variants in *TOLLIP*-KO HEK293A cells. Numerical data represent means of the relative amount of coimmunoprecipitated Venus-TOLLIP variants normalized to their expression levels in lysates ($n = 3$ independent experiments).
- D Fractionation of HEK293A cells transfected with indicated constructs into 1% Triton X-100 soluble (S) and insoluble (P) fractions. The graphs represent mean $\pm$ SEM of the relative abundance of mCherry-tagged proteins in each fraction (left) and the expression level of BiP (right) ($n = 3$ independent experiments).
- E Schematic representation of hypogonadotropic hypogonadism-linked E90K GnRHR (left) and lysosomal degradation of E90K GnRHR in HEK293A cells transfected with indicated constructs (right). The graph represents mean $\pm$ SEM of mCherry processing ($n = 3$ independent experiments).
- F Turnover of Seipin-HA-mCherry transcripts quantified by real-time quantitative PCR conducted in parallel with the experiments in Fig 7B. The data are represented as mean $\pm$ SEM ($n = 2$ independent experiments).

Data information: * $P < 0.05$, *** $P < 0.001$ by one-way ANOVA with Dunnett's multiple comparison test (E) or unpaired two-tailed Student's t test (A). †, presumed degradation products of Seipin.

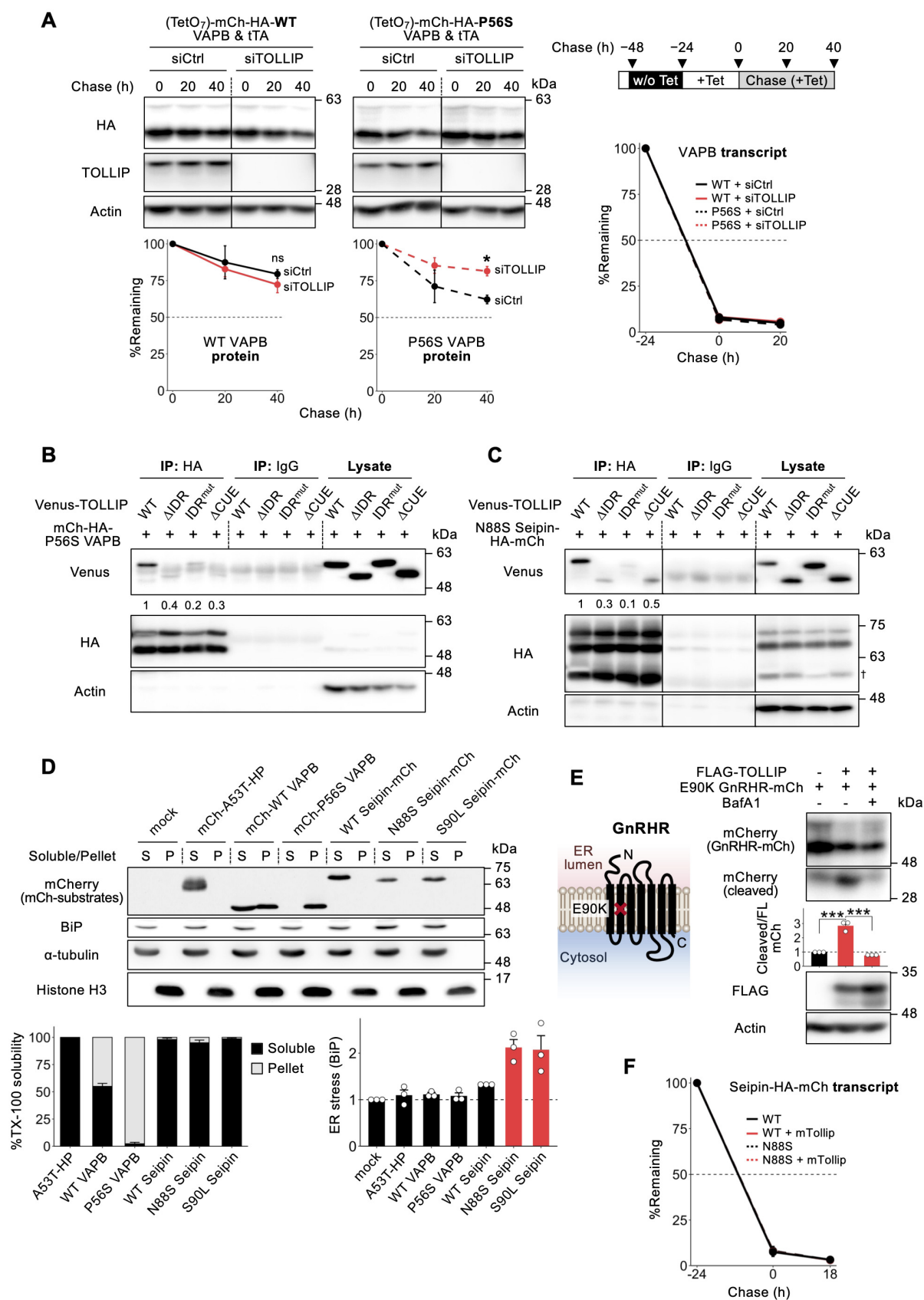


Figure EV4.

Figure EV5. TOLLIP suppresses ER stress after ERAD inhibition.

- A Quantitative PCR of *BiP* (top) and semi-quantitative PCR of *XBP1* (bottom). Ctrl KO HEK293A cells were treated with increasing concentrations of Eerl (5 or 20 μ M), NMS-873 (1.5 or 15 μ M), MG132 (0.8 or 10 μ M), or Bort (80 or 2,000 nM) for 3 h. The graph represents mean $\pm$ SEM of the *BiP* level normalized to *RPS18*, and numerical data represent means of the relative *XBP1* mRNA splicing ($n = 3$ independent experiments).
- B Immunoblot of indicated HEK293A cell lines transfected with HA-tagged null Hong Kong (NHK) variant of α 1-antitrypsin and FLAG-tagged D18G transthyretin (TTR) and treated with 5 μ M Eerl for indicated times. The graph represents mean $\pm$ SEM of Eerl-dependent fold increase in NHK and TTR levels normalized to α -tubulin ($n = 3$ independent experiments). These VCP-dependent luminal ERAD substrates accumulated to similar levels by Eerl, suggesting that Eerl treatment and subsequent blockade of ERAD were equally effective in all the cell lines analyzed.
- C Immunoblot of indicated HEK293A cell lines treated with 5 μ M Eerl for indicated times. The graph represents mean $\pm$ SEM of the IRE1 α level normalized to α -tubulin ($n = 4$ independent experiments). It had been previously shown that depletion of TOLLIP hyperactivated the ER stress sensor IRE1 α and its downstream signaling in steady-state mouse embryonic fibroblasts (MEFs) by upregulating IRE1 α protein (Pokatayev et al, 2020). However, *TOLLIP*-KO in HEK293A cells neither increased IRE1 α nor activated steady-state downstream signaling. These results suggest that the role of TOLLIP as described herein is mechanistically different from the previously reported role in regulating the IRE1 α signaling.
- D Quantitative PCR of ER stress markers. Ctrl or *TOLLIP*-KO HEK293A cells were treated with 5 μ M Eerl for 48 h. The data are represented as mean $\pm$ SEM of ER stress marker levels normalized to *RPS18* ($n = 5$ independent experiments). * $P < 0.05$, *** $P < 0.001$ by one-way ANOVA with Tukey's multiple comparison test.
- E Schematic overview showing the TOLLIP-dependent selective lysosomal degradation of aberrant ER membrane proteins in relation to other reported ER quality control pathways.

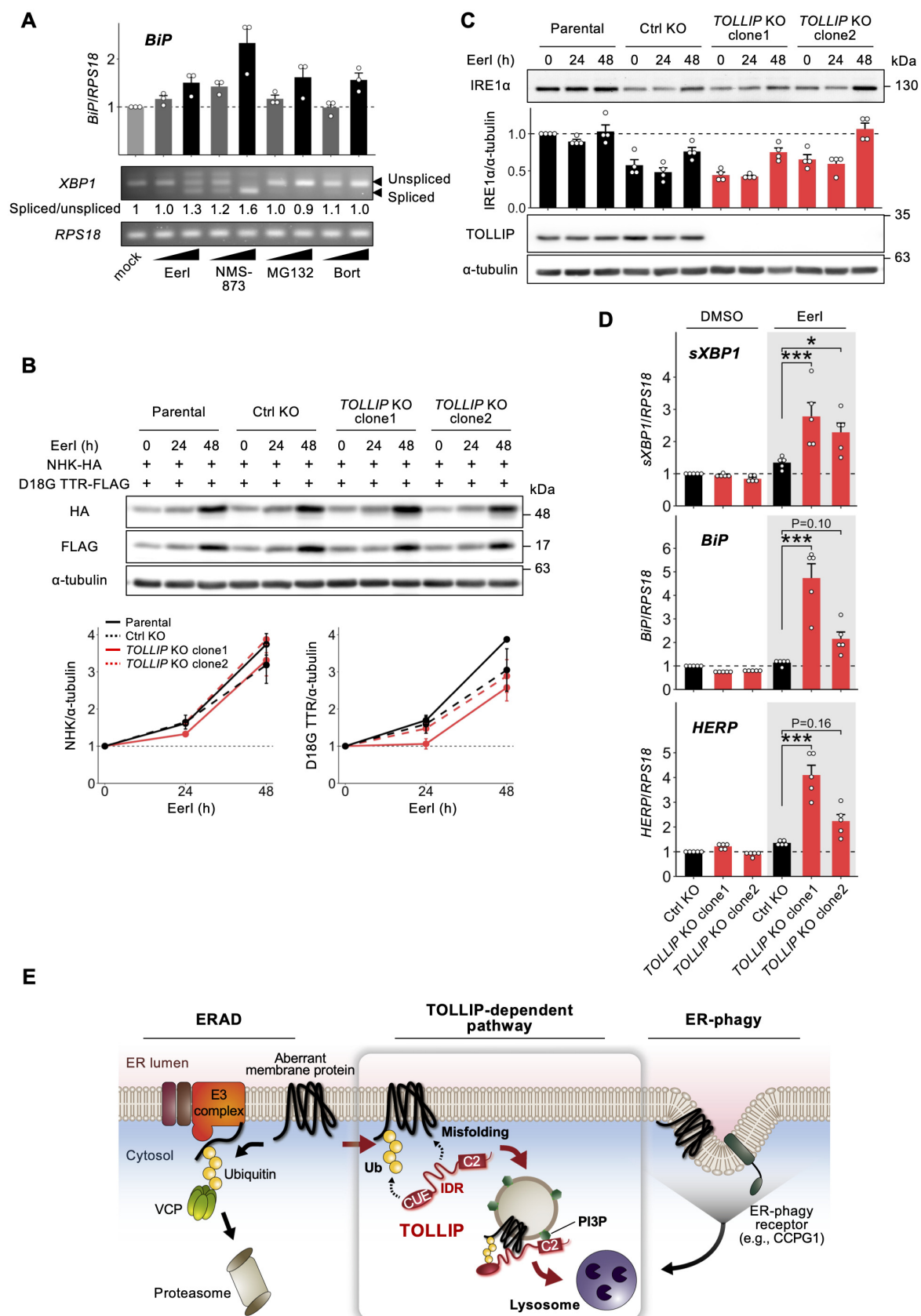


Figure EV5.

Appendix

TOLLIP acts as a cargo adaptor to promote lysosomal degradation of aberrant ER membrane proteins

Table of Contents

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Appendix Table S1. Reagents and Tools Table.

Reagent/Resource	Reference or Source	Identifier or Catalog Number
Experimental Models		
Human: HEK293 T-REx/A53T-HA-HP cells	This study	N/A
Human: HEK293A cells	Invitrogen	Cat#R70507
Human: HEK293A T-REx/mCherry-A53T-HA-HP cells	This study	N/A
Human: HEK293A T-REx/mCherry-HA-VAPB cells	This study	N/A
Human: HEK293A T-REx/mCherry-HA-P56S VAPB cells	This study	N/A
Human: HEK293A <i>CCPG1</i> KO cells	This study	N/A
Human: HEK293A <i>FAM134B</i> KO cells	This study	N/A
Human: HEK293A <i>RTN3</i> KO cells	This study	N/A
Human: HEK293A <i>SEC62</i> KO cells	This study	N/A
Human: HEK293A <i>TEX264</i> KO cells	This study	N/A
Human: HEK293A <i>FIP200</i> KO cells	This study	N/A
Human: HEK293A <i>ATG7</i> KO cells	This study	N/A
Human: HEK293A <i>TOLLIP</i> KO cells clone1	This study	N/A
Human: HEK293A <i>TOLLIP</i> KO cells clone2	This study	N/A
Human: HEK293A <i>TOLLIP</i> KO clone1/empty vector cells	This study	N/A
Human: HEK293A <i>TOLLIP</i> KO clone1/Venus-TOLLIP cells	This study	N/A
Human: HeLa cells	ATCC	Cat#CCL-2
Human: SH-SY5Y cells	ATCC	N/A
Mouse: NSC34 cells	Cedarlane	Cat#CLU140
Recombinant DNA		
pcDNA6/TR	Invitrogen	N/A

pcDNA4/TO	Invitrogen	N/A
pcDNA4/TO/A53T-HA-HP	This study	N/A
pcDNA4/TO/mCherry-A53T-HA-HP	This study	N/A
pcDNA4/TO/mCherry-A53T-FLAG-HP	This study	N/A
pcDNA4/TO/mCherry-EGFP-A53T-HA-HP	This study	N/A
pcDNA4/TO/mCherry-A53T-HA	This study	N/A
pcDNA4/TO/mCherry-E-Syt1	This study	N/A
pcDNA4/TO/VN-A53T-HA-HP	This study	N/A
pcDNA4/TO/mCherry-HA-VAPB	This study	N/A
pcDNA4/TO/mCherry-FLAG-VAPB	This study	N/A
pcDNA4/TO/mCherry-HA-P56S VAPB	This study	N/A
pcDNA4/TO/mCherry-FLAG-P56S VAPB	This study	N/A
pcDNA4/TO/Seipin-HA-mCherry	This study	N/A
pcDNA4/TO/Seipin-FLAG-mCherry	This study	N/A
pcDNA4/TO/N88S Seipin-HA-mCherry	This study	N/A
pcDNA4/TO/N88S Seipin-FLAG-mCherry	This study	N/A
pcDNA4/TO/S90L Seipin-HA-mCherry	This study	N/A
pcDNA4/TO/S90L Seipin-FLAG-mCherry	This study	N/A
pcDNA3/GW	Invitrogen	N/A
pcDNA3/GW/Venus-p62	This study	N/A
pcDNA3/GW/Venus-OPTN	This study	N/A
pcDNA3/GW/Venus-NBR1	This study	N/A
pcDNA3/GW/Venus-TOLLIP	This study	N/A
pcDNA3/GW/Venus-TOLLIP Δ TBD (Δ 1–44)	This study	N/A
pcDNA3/GW/Venus-TOLLIP TBD ^{mut} (F21A)	This study	N/A
pcDNA3/GW/Venus-TOLLIP Δ C2 (Δ 53–178)	This study	N/A

pcDNA3/GW/Venus-TOLLIP C2 ^{mut #1} (R123A)	This study	N/A
pcDNA3/GW/Venus-TOLLIP C2 ^{mut #2} (R78A)	This study	N/A
pcDNA3/GW/Venus-TOLLIP ΔIDR (Δ181–229)	This study	N/A
pcDNA3/GW/Venus-TOLLIP IDR ^{mut} (181– 229>(GGGGS) ₉ GGGG)	This study	N/A
pcDNA3/GW/Venus-TOLLIP ΔCUE (Δ231–274)	This study	N/A
pcDNA3/GW/Venus-TOLLIP CUE ^{mut} (L267A, L268A)	This study	N/A
pcDNA3/GW/Venus-TOLLIP IDR (181–229)	This study	N/A
pcDNA3/GW/FLAG-TOLLIP	This study	N/A
pcDNA3/GW/FLAG-TOLLIP ΔTBD (Δ1–44)	This study	N/A
pcDNA3/GW/FLAG-TOLLIP C2 ^{mut #1} (R123A)	This study	N/A
pcDNA3/GW/FLAG-TOLLIP C2 ^{mut #2} (R78A)	This study	N/A
pcDNA3/GW/FLAG-TOLLIP LIR ^{mut} (W133A, T134A, H135A, I136A, W151A, Y152A, S153A, L154A)	This study	N/A
pcDNA3/GW/FLAG-TOLLIP ΔIDR (Δ181–229)	This study	N/A
pcDNA3/GW/FLAG-TOLLIP IDR ^{mut} (181– 229>(GGGGS) ₉ GGGG)	This study	N/A
pcDNA3/GW/FLAG-TOLLIP ΔCUE (Δ231–274)	This study	N/A
pcDNA3/GW/FLAG-TOLLIP CUE ^{mut} (L267A, L268A)	This study	N/A
pcDNA3/GW/FLAG-NEDD4	This study	N/A
pcDNA3/GW/LAMP1-ECFP	This study	N/A
pcDNA3/GW/FLAG-C867S NEDD4	This study	N/A
pcDNA3/GW/Venus-2xFYVE	This study	N/A
pcDNA3/GW/mCherry- 2xFYVE	This study	N/A
pcDNA3/ECFP-2xFYVE	This study	N/A
pcDNA3/GW/FLAG-CFP	This study	N/A

pcDNA3/GW/VC-FLAG-TOLLIP	This study	N/A
pcDNA3/GW/H79G SAR1A-Venus	This study	N/A
pcDNA3/GW/CD147-6xMyc	This study	N/A
pcDNA3/GW/ss-mCherry-EGFP-KDEL	This study	N/A
pcDNA3/GW/FLAG-CCPG1	This study	N/A
pcDNA3/GW/FLAG-mouse Tollip	This study	N/A
pcDNA3/GW/NHK-HA	This study	N/A
pcDNA3/GW/D18G TTR-FLAG	This study	N/A
pGEX-6P-1	Cytiva	N/A
pGEX-6P-1/GST-TOLLIP IDR (181–230)	This study	N/A
pGEX-6P-1/GST-TOLLIP CUE (229–274)	This study	N/A
pGEX-6P-1/GST-HSP70	This study	N/A
pX459	(Ran <i>et al.</i> , 2013)	Addgene Plasmid #62988
pTRE-Tight-BI-DsRed-Express	Clontech	N/A
pTRE-Tight-BI-DsRed-Express/mCherry-HA-VAPB	This study	N/A
pTRE-Tight-BI-DsRed-Express/mCherry-HA-P56S VAPB	This study	N/A
pTRE-Tight-BI-DsRed-Express/Seipin-HA-mCherry	This study	N/A
pTRE-Tight-BI-DsRed-Express/N88S Seipin-HA-mCherry	This study	N/A
pTet-Off	Clontech	N/A
Antibodies		
Rat monoclonal anti-BAP31	Thermo Fisher Scientific	Cat#MA3-003; RRID: AB_325096
Goat polyclonal anti-Histone H3	Santa Cruz Biotechnology	Cat#sc-8654; RRID: AB_2118303
Mouse monoclonal anti-TOM20	Santa Cruz Biotechnology	Cat#sc-17764; RRID: AB_628381
Rabbit polyclonal anti-E-Syt1	Sigma-Aldrich	Cat#HPA016858; RRID: AB_1848404
Rat monoclonal anti- α -tubulin	Bio-Rad	Cat#MCA77G; RRID: AB_325003
Mouse monoclonal anti- β -catenin	Santa Cruz Biotechnology	Cat#sc-7963; RRID: AB_626807

Mouse monoclonal anti-SQSTM1/p62	Santa Cruz Biotechnology	Cat#sc-28359; RRID: AB_628279
Mouse monoclonal anti-KDEL (BiP)	Enzo Life Sciences	Cat#ADI-SPA-827; RRID: AB_10618036
Mouse monoclonal anti-GM130	BD Biosciences	Cat#610822; RRID: AB_398141
Mouse monoclonal anti-Actin	Sigma-Aldrich	Cat#A3853; RRID: AB_262137
Rabbit polyclonal anti-TOLLIP	Thermo Fisher Scientific	Cat#PA5-30747; RRID: AB_2548221
Rabbit polyclonal anti-OPTN	Proteintech	Cat#10837-1-AP; RRID: AB_2156665
Rabbit monoclonal anti-RAB7	Cell Signaling Technology	Cat#9367; RRID: AB_1904103
Rat monoclonal anti-HSC70	Enzo Life Sciences	Cat#ADI-SPA-815; RRID: AB_10617277
Rabbit polyclonal anti-SOD1	Enzo Life Sciences	Cat#ADI-SOD-100; RRID: AB_10616253
Mouse monoclonal anti-Ubiquitin	Santa Cruz Biotechnology	Cat#sc-8017; RRID: AB_628423
Goat polyclonal anti-Luciferase	Promega	Cat#G745A; RRID: AB_2335880
Rabbit monoclonal anti-NEDD4	Cell Signaling Technology	Cat#3607; RRID: AB_2149311
Mouse monoclonal anti-VPS34	Santa Cruz Biotechnology	Cat#sc-365404; RRID: AB_10846189
Rabbit polyclonal anti-CCPG1	Proteintech	Cat#13861-1-AP; RRID: AB_2074010
Rabbit polyclonal anti-FAM134B	Proteintech	Cat#21537-1-AP; RRID: AB_2878879
Mouse monoclonal anti-RTN3	Santa Cruz Biotechnology	Cat#sc-374599; RRID: AB_10986405
Rabbit polyclonal anti-SEC62	Bethyl	Cat#A303-981A; RRID: AB_2620330
Mouse monoclonal anti-TEX264	Santa Cruz Biotechnology	Cat#sc-100944; RRID: AB_2201887
Mouse monoclonal anti-LC3	Cosmo Bio	Cat#CAC-CTB-LC3-1-50; RRID: AB_1962479
Mouse monoclonal anti-ATG7	Santa Cruz Biotechnology	Cat#sc-376212; RRID: AB_10988418
Rabbit polyclonal anti-HERP	(Fujisawa <i>et al</i> , 2012)	N/A
Mouse monoclonal anti-IRE1 α	Santa Cruz Biotechnology	Cat#sc-390960
Rat monoclonal anti-HA	Roche	Cat#11867431001; RRID: AB_390919
Rabbit polyclonal anti-mCherry	Abcam	Cat#ab183628; RRID: AB_2650480
Mouse monoclonal anti-GFP	MBL International	Cat#M048-3; RRID: AB_591823
Mouse monoclonal anti-DYKDDDDK/FLAG (IB)	FUJIFILM Wako Shibayagi	Cat#012-22384; RRID: AB_10659717

Rabbit polyclonal anti-DYKDDDDK/FLAG (IF)	Thermo Fisher Scientific	Cat#PA1-984B; RRID: AB_347227
Mouse monoclonal anti-Myc	(Nishitoh <i>et al</i> , 2008)	N/A
Rat IgG1 isotype control	Bio X Cell	Cat#BE0290; RRID: AB_2687813
FITC-conjugated anti-mouse IgG	Jackson ImmunoResearch Labs	Cat#115-095-146; RRID: AB_2338599
Alexa Fluor 488-conjugated anti-mouse IgG	Molecular Probes	Cat#A-11029; RRID: AB_2534088
Alexa Fluor 488-conjugated anti-rat IgG	Molecular Probes	Cat#A-11006; RRID: AB_141373
Alexa Fluor 633-conjugated anti-rabbit IgG	Thermo Fisher Scientific	Cat#A-21070; RRID: AB_2535731
Alexa Fluor 633-conjugated anti-rat IgG	Thermo Fisher Scientific	Cat#A-21094; RRID: AB_2535749
HRP-conjugated anti-rabbit IgG	Cell Signaling Technology	Cat#7074; RRID: AB_2099233
HRP-conjugated anti-mouse IgG	Cell Signaling Technology	Cat#7076; RRID: AB_330924
HRP-conjugated anti-rat IgG	Cell Signaling Technology	Cat#7077; RRID: AB_10694715
HRP-conjugated anti-goat IgG	Santa Cruz Biotechnology	Cat#sc-2033; RRID: AB_631729
Oligonucleotides and other sequence-based reagents		
siRNA targeting sequence: Non-targeting: UGGUUACAUGUCGACUA A	Horizon	Cat#D-001810-01-05
siRNA targeting sequence: TOLLIP (#1): UCACGGUGGUACAGGCAA A	Horizon	Cat#J-016930-05
siRNA targeting sequence: TOLLIP #2: GGAAUAAGGUCAUCCACU G	Horizon	Cat#J-016930-07
siRNA targeting sequence: TOLLIP #3: GCAGGGCGUUGGCUAUGU G	Horizon	Cat#J-016930-08
p62 siRNA	Thermo Fisher Scientific	Cat#HSS113118
siRNA targeting sequence: OPTN:	Horizon	Cat#D-016269-01-0010

GCACGGCAUUGUCUAAAU
A

siRNA targeting sequence: NEDD4 #1: GAUCACAAUCCAGAACG A	Horizon	Cat#J-007178-07
siRNA targeting sequence: NEDD4 #2: GAACUAGAGCUUCUUAUG U	Horizon	Cat#J-007178-08
siRNA targeting sequence: VPS34: GAACAACGGUUUCGCUCU U	Horizon	Cat#J-007178-08
qPCR Primer: mCherry-HA- A53T-HP	Eurofins Genomics	Forward: AGTTCATGCGCTTCAAGGTG, Reverse: TTGGTCACCTTCAGCTTGGC
qPCR Primer: mCherry-HA- VAPB	Eurofins Genomics	Forward: CCCACAACGAGGACTACACC, Reverse: TGAATTCGAGGCTTGCATAA
qPCR Primer: Seipin-HA- mCherry	Eurofins Genomics	Forward: TTCCCTCGAGTATCCGTATGA, Reverse: CGCCCTCGATCTCGAACT
qPCR Primer: <i>BiP</i>	Eurofins Genomics	Forward: TGTTACAATCAAGGTCTATGAAG GTG, Reverse: CAAAGGTGACTTCAATCTGTGG
qPCR Primer: <i>HERP</i>	Eurofins Genomics	Forward: ATGGAGTCCGAGACCGAAC, Reverse: TTGGTGATCCAACAACAGCTT
qPCR Primer: <i>sXBP1</i>	Eurofins Genomics	Forward: TGCTGAGTCCGCAGCAGGTG, Reverse: GCTGGCAGGCTCTGGGGAAG
qPCR and semi-quantitative PCR Primer: human <i>RPS18</i>	Eurofins Genomics	Forward: TTTGCGAGTACTCAACACCAA, Reverse: GCATATCTTCGGCCCACA
qPCR Primer: mouse <i>Rps18</i>	Eurofins Genomics	Forward: CTTCCACAGGAGGCCTACAC, Reverse: TGGTGTTGAGTACTCGCAAAAT
semi-quantitative PCR Primer: <i>XBP1</i>	Eurofins Genomics	Forward: TTACGAGAGAAACTCA TGGC, Reverse: GGGTCCAAGTTGTCCAG AATGC

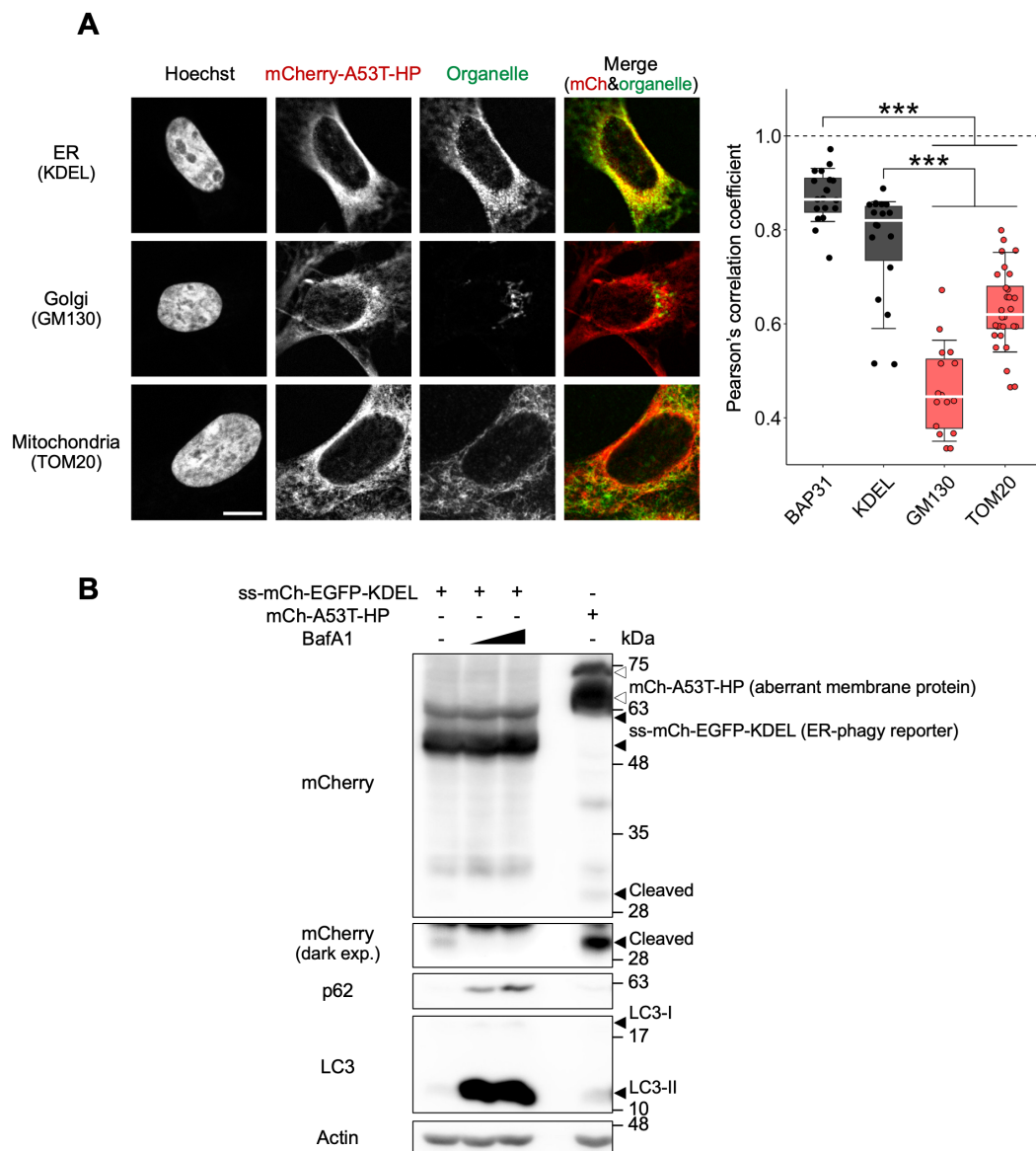
Chemicals, Enzymes and other reagents		
Polyethylenimine “MAX”	Polysciences	Cat#24765
Lipofectamine RNAiMAX	Thermo Fisher Scientific	Cat#133778500
Lipofectamine 3000	Thermo Fisher Scientific	Cat#L3000008
Hoechst 33342	Dojindo	Cat#346-07951
LysoTracker Red DND-99	Invitrogen	Cat#L7528
Tetracycline	Sigma-Aldrich	Cat#T7660
MG132	Enzo Life Sciences	Cat#BML-PI102
Bafilomycin A1	Merck Millipore	Cat#196000
Bafilomycin A1	Cayman	Cat#11038
SAR405	Med Chem Express	Cat#HY-12481
Rapamycin	Selleck	Cat#S1039
Tunicamycin	FUJIFILM Wako	Cat#202-08241
Protein G Sepharose 4 Fast Flow	Cytiva	Cat#17061802
Glutathione Sepharose 4B	Cytiva	Cat#17075601
Anti-FLAG M2-Agarose Gel	Sigma-Aldrich	Cat#A2220
Anti DYKDDDDK tag Antibody Beads	FUJIFILM Wako	Cat#016-22784
3xFLAG peptide	Sigma-Aldrich	Cat#F4799
3xFLAG peptide	M&S TechnoSystems	Cat#GEN-3-FLAG-5
QuantiLum Recombinant Luciferase	Promega	Cat#E1701
Ponceau S solution	APRO Science	Cat#SP-4030
ECL Select Western Blotting Detection Reagent	Cytiva	Cat#RPN2235
ISOGEN	Nippongene	Cat#319-90211
dNTP mix	Promega	Cat#U1515
5x Green Go Taq Reaction Buffer	Promega	Cat#M7911
Taq polymerase	This study	N/A
Software		
Fiji	(Schindelin <i>et al</i> , 2012)	https://fiji.sc
RStudio	RStudio	https://www.rstudio.com

R	The R Foundation	https://www.r-project.org
Adobe Illustrator	Adobe	https://www.adobe.com/products/illustrator.html
CHOPCHOP	(Labun <i>et al</i> , 2019)	https://chopchop.cbu.uib.no
DISOPRED3	(Jones & Cozzetto, 2015)	http://bioinf.cs.ucl.ac.uk/psipred
PONDR	(Romero <i>et al</i> , 2001)	http://www.pondr.com
SPOT-Disorder2	(Hanson <i>et al</i> , 2019)	https://sparks-lab.org/server/spot-disorder2
Other		
ReverTra Ace qPCR RT Master Mix	TOYOBO	Cat#FSQ-301
FastStart Essential DNA Green Master	Roche	Cat#06924204001
KAPA SYBR Fast qPCR Kit	Kapa Biosystems	Cat#KK4602
DC Protein Assay Reagents Package Kit	Bio-Rad	Cat#5000116

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Appendix Figure S1

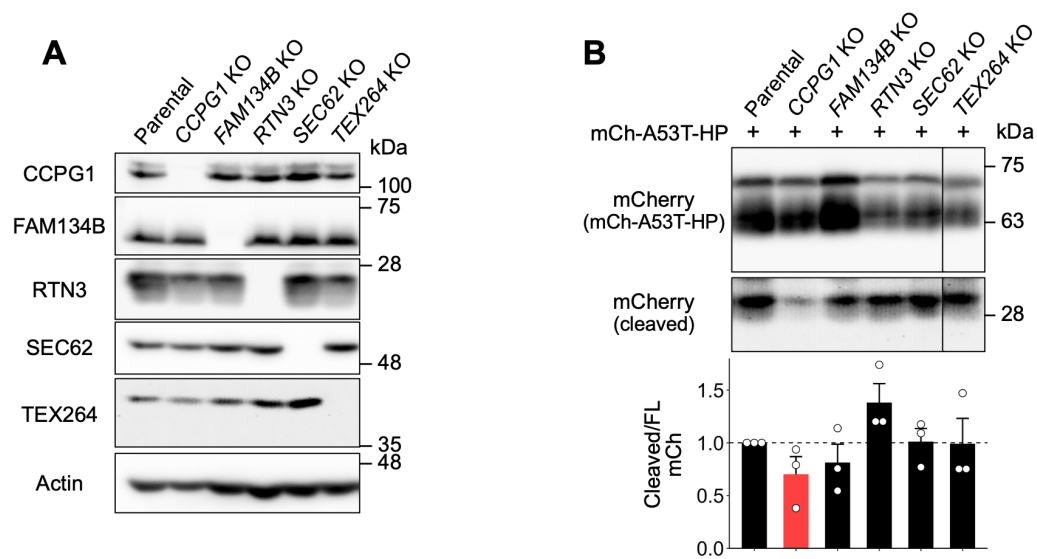


Appendix Figure S1. ER localization of a model aberrant membrane protein.

- A** Colocalization analysis of A53T-HP and organelle markers. HEK293A cells expressing tetracycline-inducible mCherry-A53T-HP were immunostained with anti-KDEL, GM130, or TOM20 antibodies. The graph represents Pearson's correlation coefficient measured between mCherry-A53T-HP and organelle markers. A total of 16–29 cells in each condition pooled from $n = 2$ independent experiments were analyzed. Box centers indicate medians, box edges represent first and third quartiles, and whiskers show the 10th to 90th percentile. Scale bar, 10 μ m. *** $P < 0.001$ by one-way ANOVA with Tukey's multiple comparison test. A representative micrograph of BAP31 staining is shown in Fig 1B.

- B Lysosomal delivery of the ER-phagy reporter ss-mCh-EGFP-KDEL and the aberrant membrane protein A53T-HP monitored by mCherry processing. HEK293A cells transfected with indicated constructs were treated with increasing concentrations of BafA1 (200 or 400 nM) for 24 h. The rightmost lane was duplicated from the leftmost lane of Fig 1E to confirm that mCherry fragments of the same size were produced from both mCherry-tagged constructs.

Appendix Figure S2



Appendix Figure S2. Involvement of ER-phagy receptors in lysosomal degradation of an aberrant ER membrane protein.

- A Validation of ER-phagy receptor KO in HEK293A cells as determined by immunoblotting.
- B Lysosomal degradation of A53T-HP in HEK293A cells with indicated genotypes transfected with mCherry-A53T-HP. The graph represents mean $\pm$ SEM of mCherry processing ($n = 3$ independent experiments).