Supplementary items for:

Hessa, T. *et al.* "Protein targeting and degradation are coupled for elimination of mislocalized proteins"

I. Technical Notes.

II. Supplementary Figures S1-S23.

I. Technical notes relating to each of the main figures.

Notes for Fig. 1.

Panel a – Translation time was 1 h at 32°C. The translated products are detected by autoradiography via the ^{35}S-Methionine incorporated into the newly synthesized protein. Ubiquitinated products are not degraded in this system due to the presence of Hemin in the lysate. Hemin inhibits both p97 activity and proteaseome activity. De-ubiquitination activity in the lysate is relatively modest, as only a small increase in ubiquitination was observed in the presence of Ubiquitin-aldehyde. His-Ubiquitin was included in the translation reactions at 10 uM. The ubiquitinated products (right panel) were isolated by immobilized Co^{+2} after complete denaturation of the translation reaction, and detected by autoradiography. Note that the N-terminus of PrP contains a copper-binding domain, contributing to a small amount of background in the Co^{+2} pulldowns in this and other experiments (i.e., some non-ubiquitinated product is also recovered).

Panel b – Time course of an experiment as in panel A without microsomes. The top panel shows an autoradiograph of ubiquitinated PrP, isolated via the His-Ubiquitin. The bottom panel shows full length PrP detected by autoradiography of total translation products. Note that ubiquitination occurs very shortly after synthesis. This is different than a tail-anchored protein destined for post-translational insertion into the ER, where ubiquitination lags behind synthesis by ~10-20 min (see Sup. Fig. S23).

Panel c – In vitro synthesized transcripts were generated of the complete PrP coding region (residues 1-254). The 'terminated' transcript contains a stop codon plus ~200 nucleotides of a 3'UTR, while the 'truncated' transcript ends precisely at the last codon (and therefore does not contain an in-frame stop codon). This results in a peptidyl-tRNA that remains tethered to the ribosome at the P-site, and is generally stable for the ~1 h time-frame of the experiment. Analysis of the truncated product on SDS-PAGE shows some tRNA-associated product, which is partially hydrolyzed during sample preparation and electrophoresis under the slightly basic conditions. The identity of the band (arrowhead) as tRNA-associated was verified by RNAse digestion just before electrophoresis. The ribosome-nascent chains of truncated PrP were isolated by sucrose gradient centrifugation (to remove bulk cytosol) and treated with 1 mM puromycin for 1 h to release the chains. The release reactions contained an energy regenerating system, His-Ubiquitin, E1 enzyme, and E2 enzyme. One reaction also contained RRL (cytosol), while the other contained a matched buffer. Note that cytosol is needed to get ubiquitination of substrate.

Panel d – Both the signal sequence and GPI anchoring sequence are hydrophobic, and both contribute to the overall ubiquitination efficiency of PrP. The precise sequence of the signal was not important since different signal sequences of varying amino acid composition and sequence facilitated ubiquitination when used to replace the PrP signal sequence.

Notes for Fig. 2.

Panel a – The Fr-RRL system is essentially a translation-competent reaction corresponding to the classic 'Fraction II' from the original ubiquitination studies of Hershko, Ciechanover, and Rose.

This fraction does not contain much ubiquitin, hence requiring supplementation. Ubiquitin supplementation however is not enough to complement ubiquitination of MLPs, even though ubiquitination of other proteins is restored (see Sup. Fig. S7). This is because in addition to ubiquitin, subsets of E2s and E3s are also missing from the reaction.

Panel b – Translation time was for 1 h at 32°C in the Fr-RRL system without or with 250 nM UbcH5a. All reactions contained 10 uM His-Ubiquitin. For post-translational ubiquitination, UbcH5a was added after the translation, and incubated for another hour at 32°C. Ubiquitinated products were isolated by immobilized Co^{+2}, while total products were analyzed directly, and detected by autoradiography. The fact that UbcH5a can be added after translation means that the PrP substrate is being maintained in a ubiquitination-competent state after its synthesis, and that it does not need to be ubiquitinated immediately.

Panel c - For immunoaffinity purification, the PrP construct was appended with an HA epitope, inserted at residue 50 in the flexible N-terminal domain. After translation, PrP-HA was purified under native, non-detergent conditions, using immobilized anti-HA antibodies. The absence of detergent is important because the hydrophobic interaction between PrP and Bag6 is sensitive to detergent (but relatively insensitive to salt). The beads were washed in detergent-free buffer and the ubiquitination reaction performed directly on the beads. Ubiquitination was for 1 h at 32°C. After the reaction, the samples were completely denatured and the ubiquitinated products isolated via immobilized Co^{+2}. The fact that cytosol is not needed suggests that the PrP pulldowns contain an active E3 enzyme.

Panel d – PrP-HA was used for this experiment to facilitate the ubiquitination assays. Chemical crosslinking was with BMH at 250 uM on ice for 30 min. Similar results were seen with DSS crosslinking, but typically generated more background. For the ubiquitination assays, each fraction was affinity purified via the HA tag as in panel c and subjected to ubiquitination on the beads. The relative ubiquitination was quantified and graphed.

Panel e – After translation of the indicated constructs, the samples were separated on a sucrose gradient as in panel d, and fractions 6-10 were pooled for the crosslinking reactions.

Notes for Fig. 3.

Panel a – The extra TMD inserted into Sec61β was from Transferrin Receptor (TR), which has been showed experimentally to interact with SRP as a nascent chain. The control hydrophilic sequence is the random sequence encoded by the same oligos inserted in the reverse orientation. The mutation within the TMD of Sec61β introduces three Arginines, and this has been shown to disrupt binding to the TA insertion machinery, including Bag6 and TRC40.

Panels b and c – Crosslinking was with DSS at 200 uM for 30 min at 25°. The same results were obtained with BMH crosslinking for the Bag6 interaction, but BMH does not effectively crosslink SRP, presumably because of a lack of suitably positioned cysteine residues.

Panel d – All proteins were full length (i.e., not to be confused with the RNCs in panels b and c). Affinity purification was performed via anti-Sec61β antibodies under detergent-free conditions

to preserve the hydrophobic interactions between substrates and Bag6 and/or TRC40.

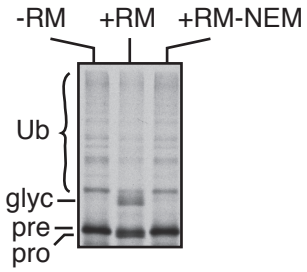
Notes for Fig. 4.

Panel a – Translation times were for 1 h at 32°C. Reactions contained 10 uM His-Ubiquitin to allow purification of the ubiquitinated products. Immunodepletion was via an anti-Bag6 antibody column, and the non-depleted control used an anti-GFP antibody column. Depletion efficiency was judged by immunoblotting to be ~90%. Depletion of Bag6 results in at least 80% co-depletion of its associated factors Ubl4A and TRC35.

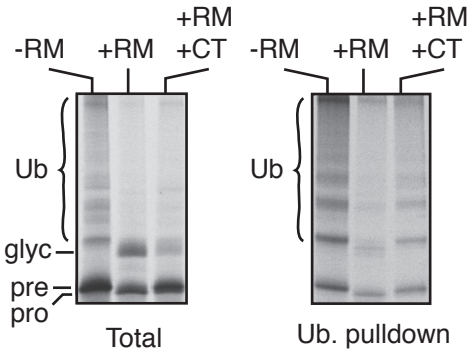
Panel b – The purified Bag6 and ΔUbl-Bag6 did not contain its associated factors Ubl4A and TRC35, while the native Bag6 complex did. Relative levels were assessed by immunoblotting of serial dilutions.

Panel c – Crosslinking reactions were performed as in Figure 2e. The top panel shows high molecular weight crosslinking products. The identity of the band as a Bag6 crosslink was verified by both anti-Bag6 and anti-FLAG IPs. As expected, the recombinant Bag6 and ΔUbl-Bag6 were immunoprecipitated with anti-FLAG and anti-Bag6, while the endogenous Bag6 was only immunoprecipitated with anti-Bag6.

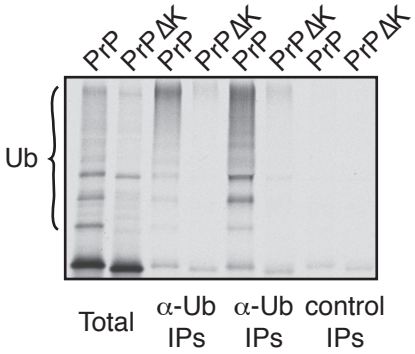
Panels d and e – Cells were harvested ~22-24 h after transfection. The increase in N3a-PrP levels seen with ΔUbl-Bag6 or with proteasome inhibitor is due to decreased degradation, and not increased synthesis since control proteins (e.g., GFP) were verified to be unaffected in their levels under identical conditions. Similarly, the matched construct lacking hydrophobic elements (ΔSSΔGPI-PrP) was also unaffected in its levels by ΔUbl-Bag6, illustrating that ΔUbl-Bag6 does not affect protein production.



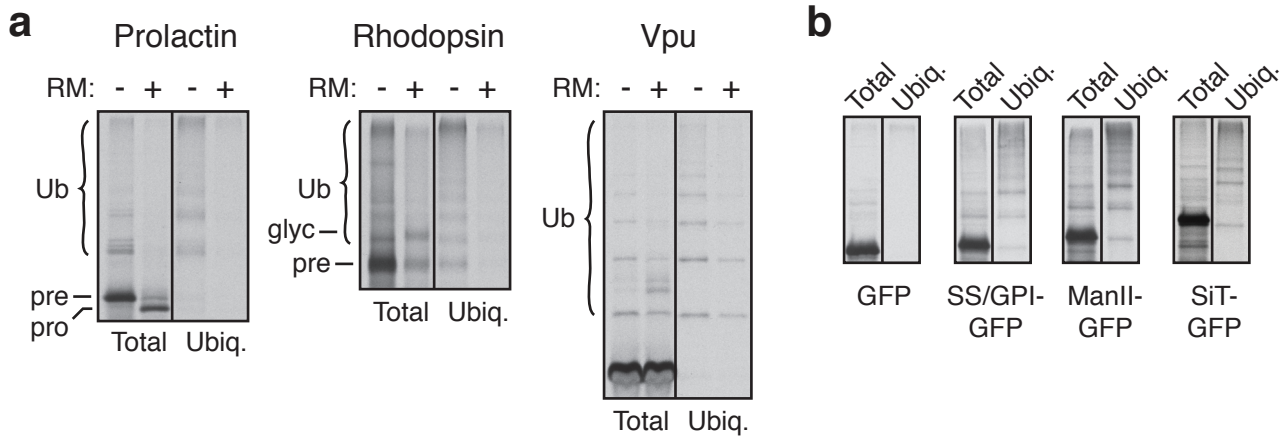
Sup. Fig. S1. PrP that fails targeting is ubiquitinated in vitro. PrP was translated for 1 hour in reticulocyte lysate lacking or containing mock-treated or NEM-treated ER-derived rough microsomes (RMs). Treatment of RMs was with 0 or 3 mM N-ethyl maleimide (NEM) for 15 min at 25°C. DTT was then added to 10 mM to inactivate NEM, and the microsomes were re-isolated by centrifugation prior to use in the assay. Note that these manipulations lead to a slight loss of targeting activity of the control RMs, while NEM-treated RMs are completely inactive due to alkylation of the SRP-receptor. The PrP that fails targeting to the NEM-inactivated RMs is ubiquitinated.



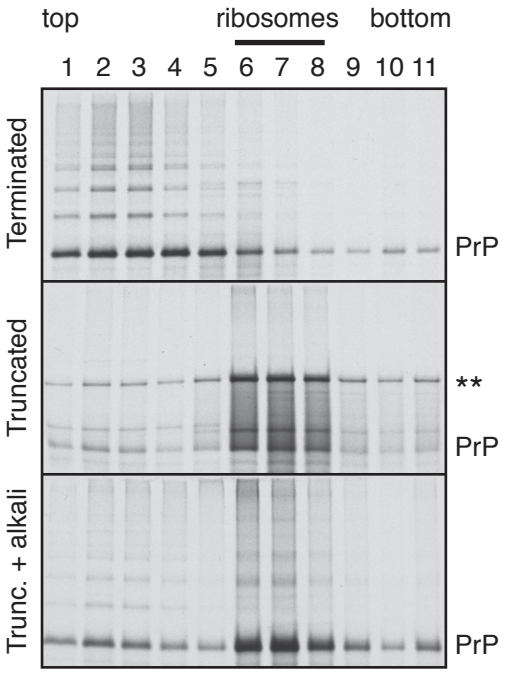
Sup. Fig. S2. PrP that fails translocation is ubiquitinated. PrP was translated in reticulocyte lysate lacking or containing ER-derived rough microsomes (RMs), without or with the translocation inhibitor cotransin (CT, added to 10 uM; Garrison et al., 2005, Nature, 436:285-9). Translation reactions contained 10 uM His-ubiquitin. After translation, an aliquot was analyzed directly (left panel; 'Total'), while the remainder was pulled down with immobilized Co^{+2} to recover ubiquitinated products (right panel; 'Ub pulldown'). Note that PrP is only ~50% inhibited in its translocation by cotransin. The positions of precursor, processed, glycosylated, and ubiquitinated forms of PrP are indicated. Translation time was 1 h.



Sup. Fig. S3. Lysines in PrP are needed for ubiquitination. PrP and a construct with all Lysines mutated to Arginines (PrPΔ K) were translated in reticulocyte lysate for 1 h. An aliquot was analyzed directly ('Total'), while the remainder was immunoprecipitated with two different anti-Ubiquitin antibodies or an unrelated control antibody. Re-introduction of a single Lysine into PrP was sufficient to restore ubiquitination (unpublished observations).

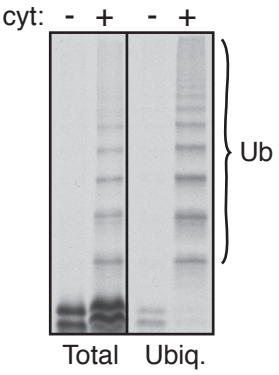


Sup. Fig. S4. Analysis of various proteins for ubiquitination in vitro. (a) The indicated proteins were translated in reticulocyte lysate (supplemented with 10 uM His-ubiquitin) without or with RM. An aliquot was analyzed directly ('Total'), while the remainder was pulled down with immobilized Co^{+2} to enrich for ubiquitinated species ('Ubiq.'). Prolactin is a secretory protein with an especially hydrophobic signal sequence; Rhodopsin is a 7-TM domain protein; Vpu is type I signal anchored membrane protein (from HIV). (b) The indicated proteins were translated in reticulocyte lysate supplemented with 10 uM His-ubiquitin. An aliquot was analyzed directly ('Total'), while the remainder was pulled down with immobilized Co^{+2} to enrich for ubiquitinated species ('Ubiq.'). SS/GPI-GFP contains the N-terminal signal sequence from Prolactin and C-terminal GPI anchoring signal from PrP; SiT-GFP and ManII-GFP contains the Type I signal anchor sequences from Sialyl transferase and Golgi Mannosidase II, respectively, at the N-terminus. Note that GFP is poorly ubiquitinated, but becomes a much better target when appended with targeting/translocation signals.

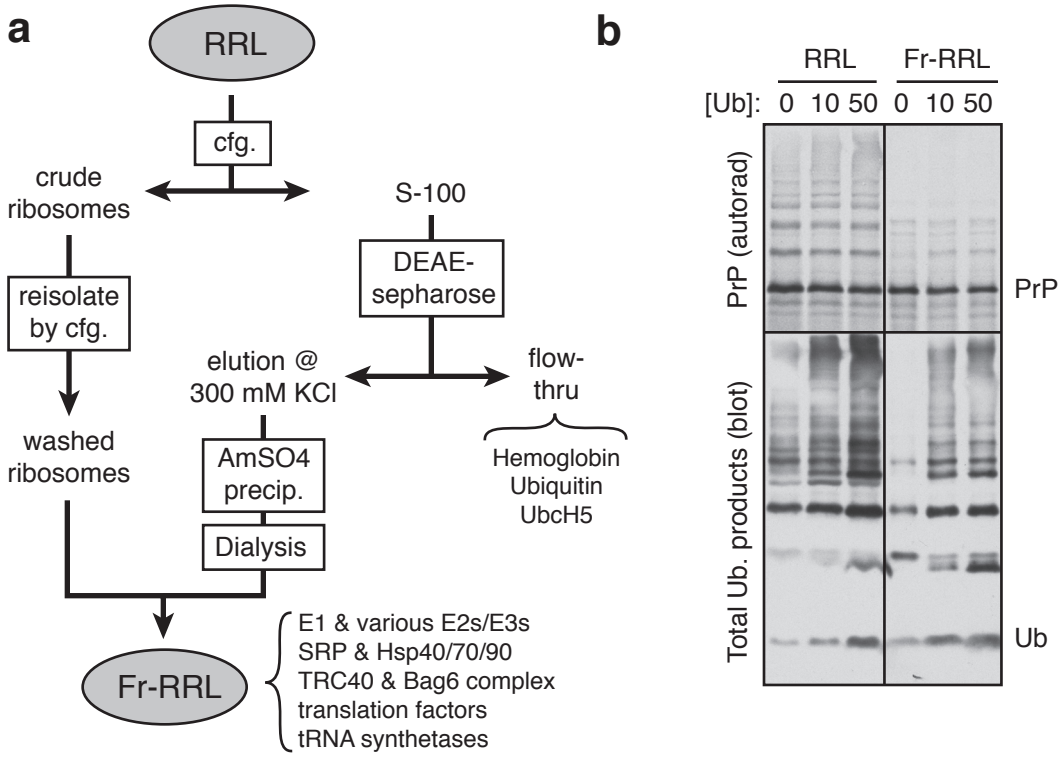


Sup. Fig. S5. PrP is ubiquitinated after ribosomal release. PrP transcript containing (terminated) or lacking (truncated) a stop codon was translated in reticulocyte lysate, separated on a 10-50% sucrose gradient, and each of 11 fractions analyzed by SDS-PAGE. The samples in the bottom gel were adjusted to pH 11 before electrophoresis to hydrolyze tRNA, while the samples in the middle gel were maintained under near-neutral conditions to preserve the peptidyl-tRNA species (indicated with asterisks). The position of ribosomes in the gradient is indicated.

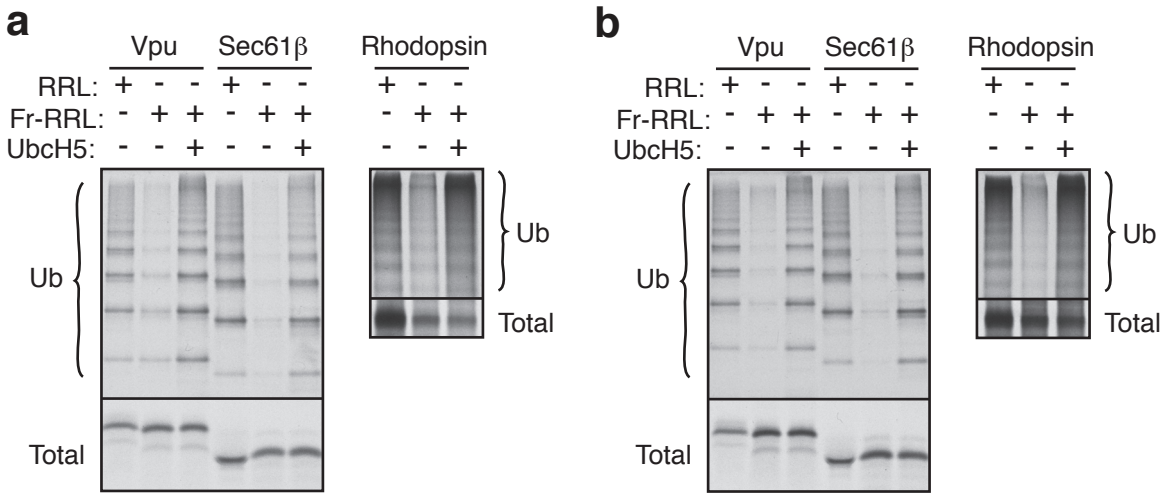
Note that little or no ubiquitination is seen in the ribosomal fraction of truncated PrP nascent chains. The only ubiquitination observed in the truncated sample appears to arise from spontaneously released polypeptides. The smear seen in the ribosomal fractions is not likely to be ubiquitination since it could not be recovered by anti-ubiquitin antibodies (not shown), and may instead represent some PrP aggregation.



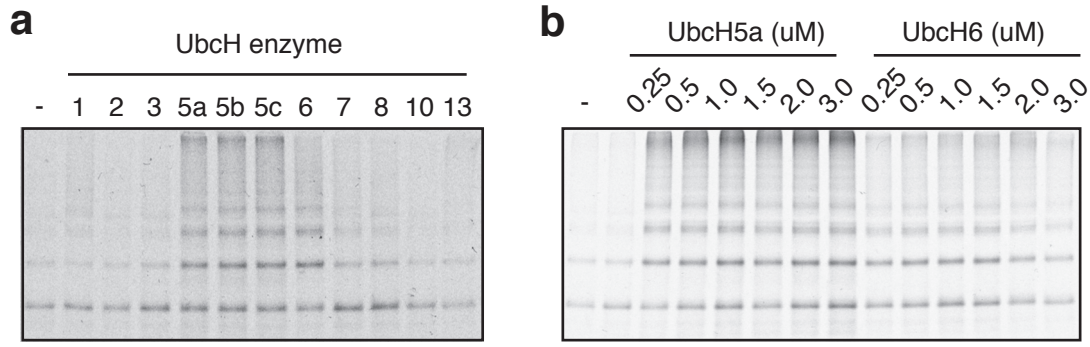
Sup. Fig. S6. Vpu becomes ubiquitinated upon release into the cytosol. Ribosome-nascent chains encoding Vpu (an 81-residue polypeptide, with the first ~20 residues encoding a TM domain) were isolated from a sucrose gradient (e.g., as in Sup. Fig. S5) and released with puromycin in the presence of an energy regenerating system, E1 (100 nM), UbcH5 (250 nM), and His-ubiquitin (10 uM), without or with cytosol ('cyt.'). After incubation for 60 min, the samples were either analyzed directly ('Total') or after pulling down ubiquitinated products with immobilized Co^{+2} ('Ubiq'). The experiment in Fig. 1c was performed exactly the same way.



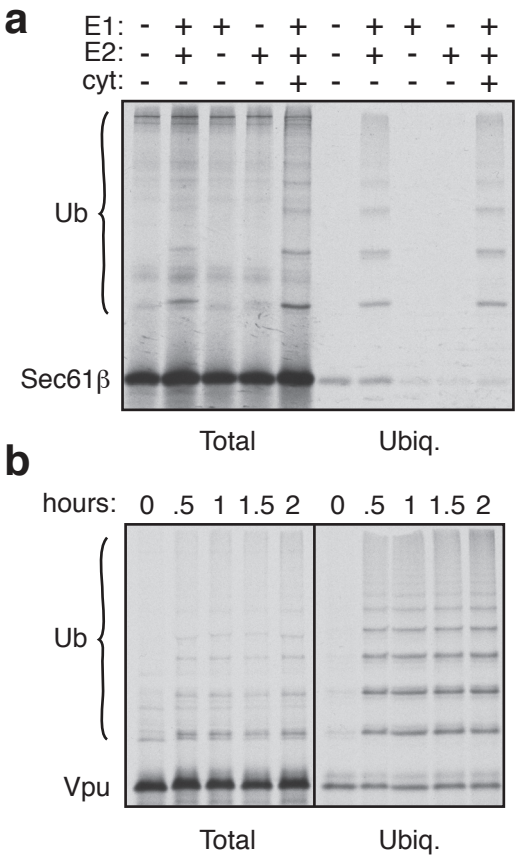
Sup. Fig. S7. A fractionated translation system from RRL. (a) Schematic diagram of the fractionation procedure to produce Fr-RRL. Ribosomes are removed from RRL by centrifugation, washed, and added back to a DEAE-elution fraction. This system, when complemented with tRNAs, amino acids, and an energy regenerating system, is competent for translation. Indicated is a partial list of key factors verified (by blots and/or activity) to be in Fr-RRL, and those known to be lost in the DEAE flow thru fraction. (b) PrP was translated in either RRL or Fr-RRL supplemented with the indicated concentrations (in uM) of His-ubiquitin. The translation products were separated by SDS-PAGE, transferred to nitrocellulose, and visualized by autoradiography to detect the translated PrP (top panel; the image is the same as that in Fig. 2a). This same blot was also probed with anti-ubiquitin to detect endogenous ubiquitinated species (bottom panel). This illustrates that the Fr-RRL system, when supplemented with ubiquitin, is competent for ubiquitination of various endogenous proteins, but is substantially impaired for ubiquitination of non-translocated PrP.



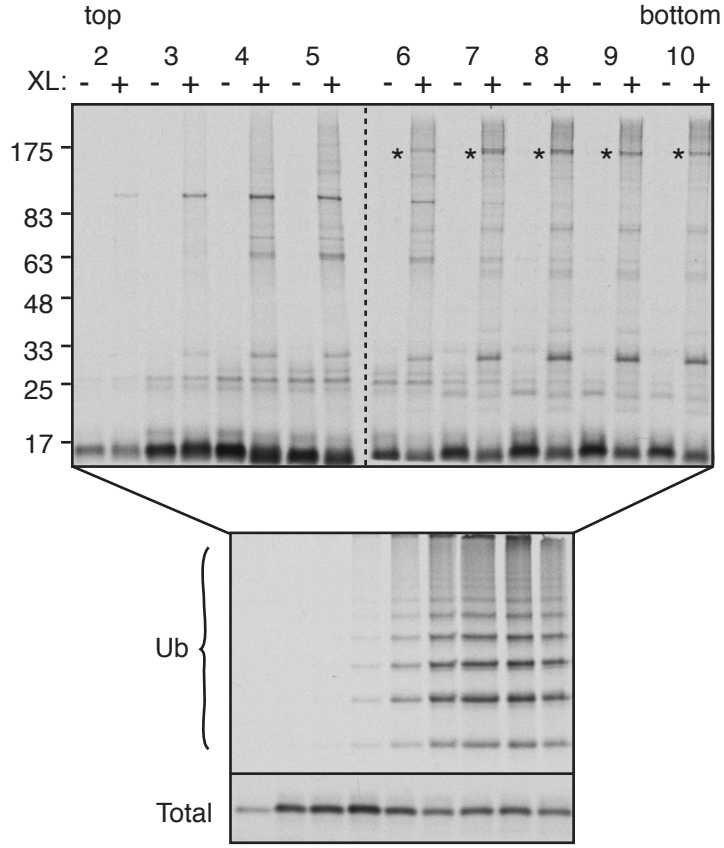
Sup. Fig. S8. Various MLPs require UbcH5 for ubiquitination in vitro. Ubiquitination assays for various MLPs were performed in RRL and Fr-RRL similarly to Fig. 2a and 2b. The indicated constructs were translated in either RRL or Fr-RRL supplemented with 10 uM His-ubiquitin. Where indicated, 250 nM UbcH5 (isoform a) was included co-translationally (panel a) or added after translation (panel b). In the co-translational reactions, total translation time was 1 h. In the post-translational reaction, translation was also for 1 h, but the incubation continued for an additional 1 h after UbcH5 was added. The samples were either analyzed directly ('Total') or after pulling down ubiquitinated products with immobilized Co^{+2} (top panels). Note that some proteins (e.g., Rhodopsin) do not translate as well in Fr-RRL as in RRL for unclear reasons. Nonetheless, it is apparent that upon replenishment of Fr-RRL with UbcH5 (either co- or post-translationally), levels of ubiquitination are comparable to RRL, suggesting that this was the main if not only factor missing in Fr-RRL needed for this pathway.



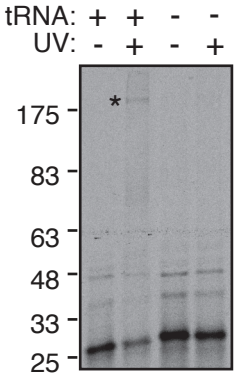
Sup. Fig. S9. Analysis of various E2 enzymes for ability to restore ubiquitination activity to Fr-RRL. (a) Ribosome-nascent chains of PrP were released with puromycin into Fr-RRL supplemented with 10 uM His-ubiquitin, 100 nM E1 enzyme, and an ATP regenerating system. The indicated E2 enzyme was included at 3 uM. After 1 h at 32°C, the ubiquitinated products were captured with immobilized Co^{+2} and analyzed by SDS-PAGE. UbcH5 isoforms and highly related UbcH6 enzyme have significant activity, while the others are less active. (b) An experiment as in panel a comparing UbcH5a to UbcH6 at various concentrations. Note that UbcH5a is highly active even at the lowest concentration (250 nM), while UbcH6 was substantially less active at all concentrations. Furthermore, immunoblotting of fractions during the Fr-RRL preparation confirmed that UbcH5 was lost during the ion exchange step and therefore not present in the final Fr-RRL sample.



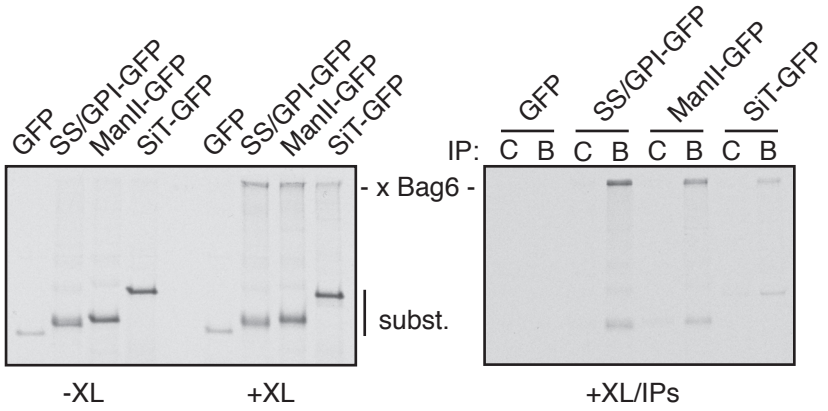
Sup. Fig. S10. Ubiquitination of affinity purified Sec61β and Vpu with purified components. (a) Sec61β was translated in Fr-RRL and immunoaffinity purified under non-denaturing, non-detergent conditions using an anti-Sec61β antibody resin. After washing of the resin, the indicated components were added: 100 nM E1, 250 nM UbcH5, or cytosol. All reactions contained 10 uM His-ubiquitin and an ATP-regenerating system (1 mM ATP, 10 mM Creatine phosphate, 40 ug/ml Creatine Kinase). After incubation for 1 h at 32°C, the samples were either analyzed directly ('Total') or after pulling down ubiquitinated products with immobilized Co^{+2} ('Ubiq'). The experiment in Fig. 2c was performed exactly the same way. (b) HA-tagged Vpu was translated in Fr-RRL. Anti-HA antibodies were added, and after 30 min on ice, the antibody complexes were recovered under non-denaturing, non-detergent conditions using immobilized Protein A. The washed beads were then supplemented with 100 nM E1, 250 nM UbcH5, 10 uM His-ubiquitin and an ATP-regenerating system, and incubated for the indicated times at 32°C. The samples were either analyzed directly ('Total') or after pulling down ubiquitinated products with immobilized Co^{+2} ('Ubiq'). Note that not every protein incubated as above gets ubiquitinated, since a Sec61β construct lacking its TMD was not ubiquitinated (data not shown), and sub-populations of Vpu or PrP are not ubiquitinated under these same conditions (e.g., Fig. 2d and Sup. Fig. S11).



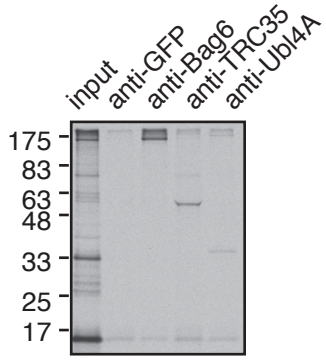
Sup. Fig. S11. Correlation of p150 crosslinking with ubiquitination of Vpu. Vpu-HA was translated in Fr-RRL and separated into 10 fractions on a 5-25% sucrose gradient. Each fraction was subjected to chemical crosslinking with 250 uM bis-maleimido-hexane (BMH), a sulfhydryl-reactive crosslinker, and analyzed by SDS-PAGE (top gel). The position of a ~150 kD crosslinking partner is indicated with an asterisk. In parallel, Vpu-HA from each fraction was affinity purified with immobilized anti-HA and assayed for ubiquitination competence by addition of E1, E2, His-ubiquitin, and energy (as in Sup. Fig. S10 above). An aliquot of the total recovered material ('Total') and ubiquitinated products (captured via the His-ubiquitin) is shown. Note that ubiquitination activity correlates with the p150 crosslink. Note also that Vpu recovered from some fractions (e.g., 2-5) is not ubiquitinated effectively, illustrating that the E1 and E2 enzymes are not by themselves sufficient to ubiquitinate Vpu.



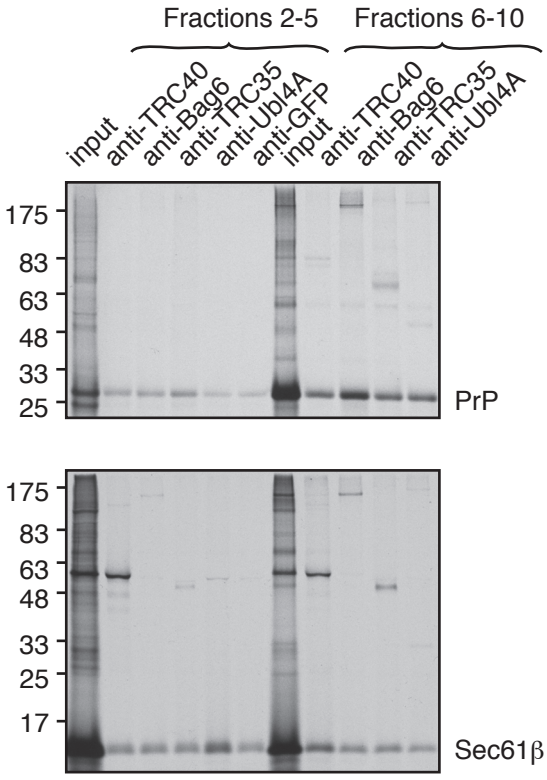
Sup. Fig. S12. Photo-crosslinking of PrP to p150. PrP was translated in Fr-RRL containing or lacking a benzophenone-modified Lysyl-tRNA. The translation products were then irradiated with UV light to induce photo-crosslinking, and analyzed by SDS-PAGE. The asterisk indicates the position of a ~150 kD crosslinking product.



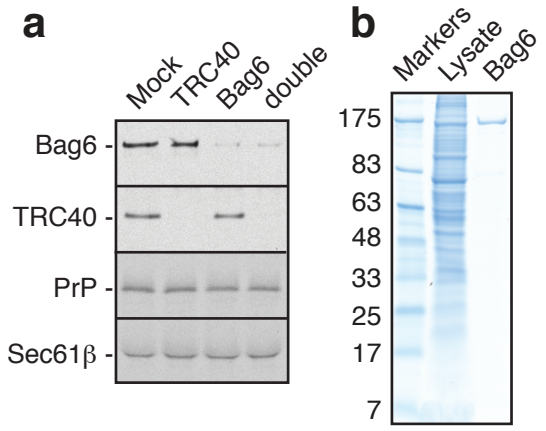
Sup. Fig. S13. Bag6 interacts with hydrophobic domains. GFP or versions appended with the indicated hydrophobic domains were translated in vitro, separated by a 5-25% sucrose gradient as in Fig. 2d, and the pooled fractions 6-10 subjected to chemical crosslinking with BMH. The samples before and after crosslinking are shown in the left panel. The right panel shows the crosslinking products immunoprecipitated with either control (C) or Bag6 (B) antibodies. 'SS/GPI' refers to the signal sequence and GPI anchoring sequence from Prolactin and PrP, respectively. 'ManII' refers to the TMD of Golgi Mannosidase II. 'SiT' refers to the cytosolic and TMD regions of Golgi Sialyl-transferase.Note that modifying GFP with hydrophobic targeting elements results in Bag6 interaction.



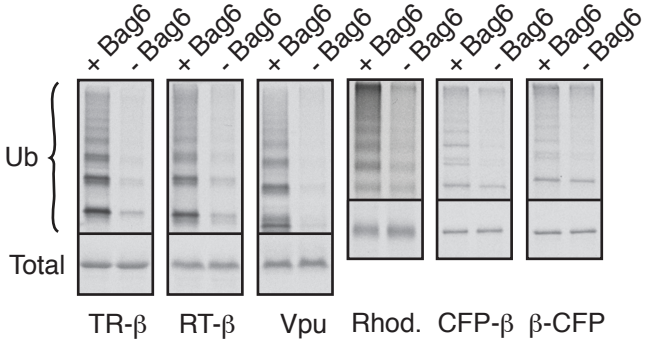
Sup. Fig. S14. Vpu crosslinking to the Bag6 complex. Vpu was translated in Fr-RRL, separated on a 5-25% sucrose gradient (as in Sup. Fig. S11), and fractions 6-10 pooled. The pooled sample was treated with BMH crosslinker on ice for 30 min, and either analyzed directly ('input') or subjected to immunoprecipitation with the indicated antibodies. As observed before (Mariappan et al., 2010, Nature, 466:1120-4), Ubl4A crosslinks only weakly to substrate, probably because it does not make direct contact with substrate but is in the vicinity due to its being part of the Bag6 complex. Affinity purification of HA-tagged Vpu (without cross-linking, under native conditions) confirmed its co-precipitation with all three subunits of the Bag6 complex by immunoblotting (not shown).



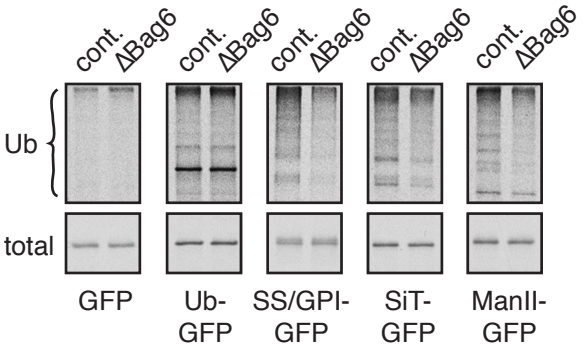
Sup. Fig. S15. PrP and Sec61β crosslinking to the Bag6 complex. PrP and Sec61β were translated in Fr-RRL, separated on a 5-25% sucrose gradient (as in Sup. Fig. S11), and fractions 2-5 and 6-10 were pooled. The pooled samples were treated with BMH crosslinker on ice for 30 min, and either analyzed directly ('input') or subjected to immunoprecipitation with the indicated antibodies. Note that both PrP and Sec61β crosslink to all three components of the Bag6 complex (Ubl4A only weakly). However, the major crosslink for Sec61β was TRC40, as observed before (Stefanovic and Hegde, 2007, Cell, 128:1147-59), consistent with the fact that Sec61β bound to the Bag6 complex is transferred to TRC40 (Mariappan et al., 2010, Nature, 466:1120-4). By contrast, PrP crosslinking to TRC40 was minimal, with Bag6 being the primary interacting partner.



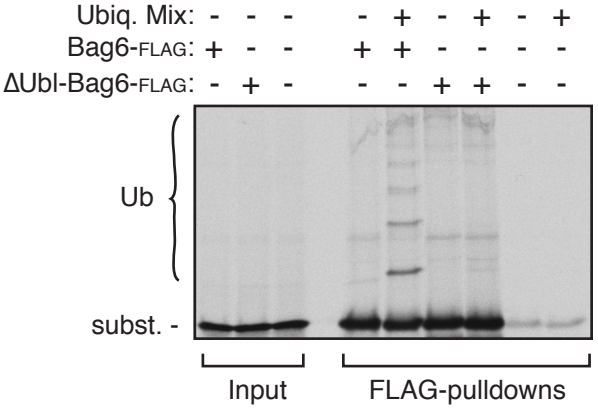
Sup. Fig. S16. Bag6 depletion and purification. (a) RRL was subjected to immunodepletion using antibodies to GFP ('Mock'), TRC40, Bag6, or both TRC40 and Bag6 ('double'). Equal aliquots of the depleted lysates were analyzed by immunoblotting against TRC40 and Bag6. Depletion of TRC40 was ~90% and Bag6 ~80%. Note that upon Bag6 depletion, Ubl4A and TRC35 are comparably depleted (Mariappan et al., 2010, Nature, 466:1120-4). In addition, the four lysates were used for in vitro translation of PrP and Sec61β to illustrate no effect on protein synthesis. Translation time was 30 min. (b) FLAG-tagged Bag6 was purified from overexpressing HEK-293T cells and analyzed by colloidal coomassie blue staining. A version lacking the Ubl domain was purified the same way with identical yield and purity (not shown).



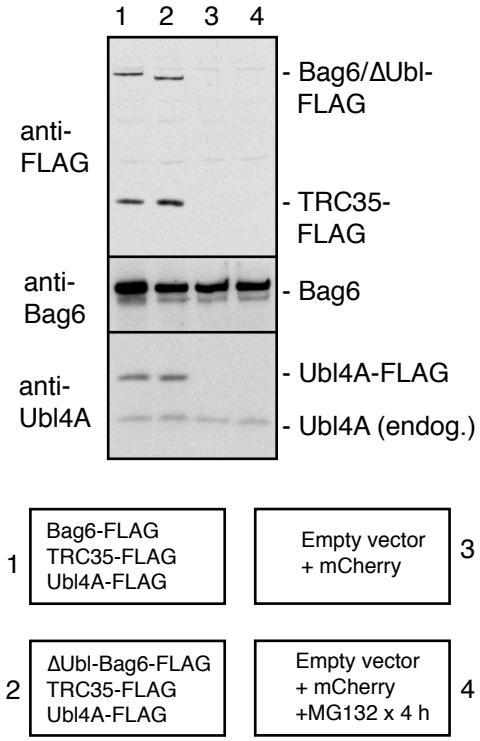
Sup. Fig. S17. Various MLPs are dependent on Bag6 for efficient ubiquitination. RRL containing or depleted of Bag6 complex was used for translations of the indicated proteins. Translation reactions contained 10 uM His-Ub, and were incubated for 1 h. Ubiquitinated products were captured using immobilized Co^{+2} (upper panels). An aliquot of total translation was also analyzed to confirm equal synthesis (bottom panels). CFP-β and β-CFP are constructs in which Sec61β was appended with CFP at the N- or C-terminus, respectively. Thus, β-CFP contains an internal TM domain, while CFP-β remains a tail-anchored protein.



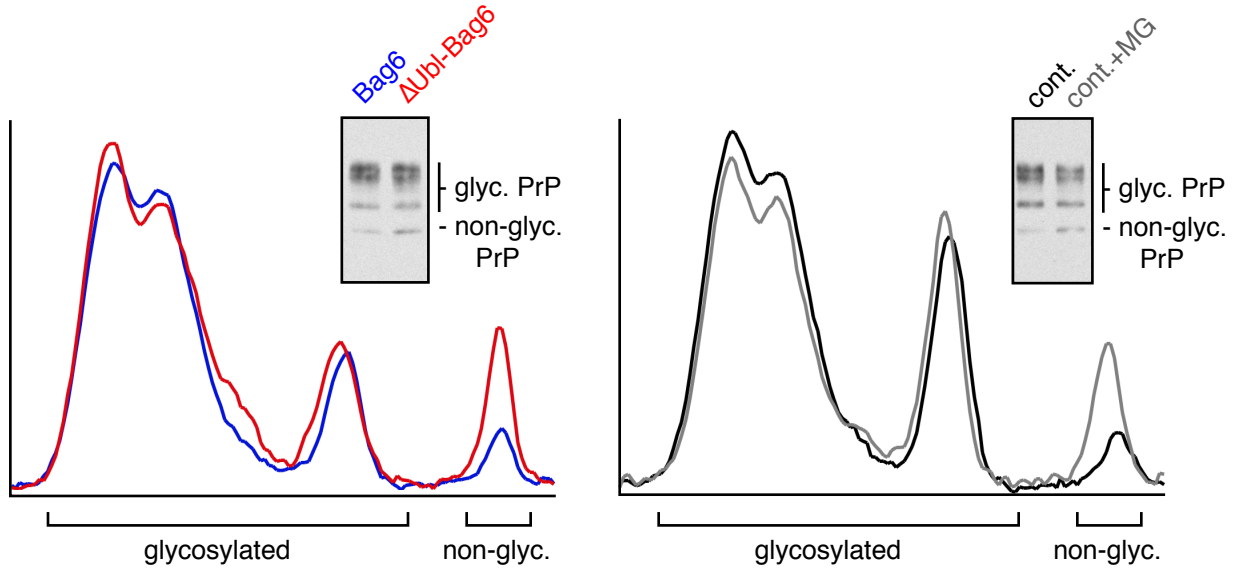
Sup. Fig. S18. The Bag6 complex facilitates ubiquitination via hydrophobic domains. GFP or versions appended with the indicated domains were translated in vitro using lysates subjected to control or Bag6 immunodepletion. 10 uM His-ubiquitin was included in the reactions. The isolated ubiquitinated products (top panels) and an aliquot of total translation products (bottom panels) are shown. Note that Bag6 depletion influences ubiquitination selectively of constructs containing hydrophobic domains. Ub-GFP is a linear fusion of Ubiquitin and GFP.



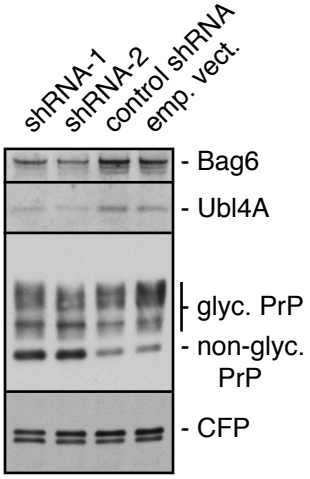
Sup. Fig. S19. The Bag6 complex recruits ubiquitination machinery via its Ubl domain. TR-β was translated in Fr-RRL supplemented with FLAG-tagged recombinant Bag6, ΔUbl-Bag6, or buffer. In the Fr-RRL system, substrate is not ubiquitinated due to lack of ubiquitin and UbcH5. The sample was affinity purified via the FLAG tag and incubated with or without a ubiquitination mix (20 uM His-ubiquitin, 100 nM E1, 250 nM UbcH5a, and energy regenerating system). An aliquot of the input translation shows equal substrate synthesis. Both Bag6 and ΔUbl-Bag6 effectively capture substrate, but only the former becomes ubiquitinated (verified by ubiquitin pulldowns; not shown).



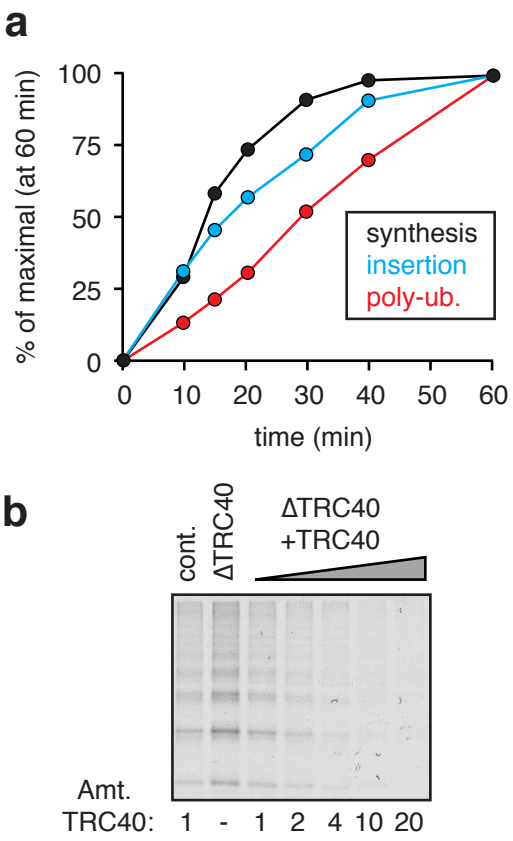
Sup. Fig. S20. Characterization of Bag6 complex expression. Mouse N2a cells in a 24-well dish were transfected with a mixture of expression plasmids for Bag6-FLAG (425 ng), TRC35-FLAG (225 ng), Ubl4A-FLAG (50 ng) and a PrP construct (100 ng). Preliminary experiments established the relative ratios that gave roughly equal expression levels of each Bag6 complex subunit. Lane 2 contained ΔUbl-Bag6-FLAG instead of Bag6-FLAG. Lanes 3 and 4 contained an empty vector (600 ng) and 100 ng mCherry expression vector in lieu of Bag6 complex plasmids. At 20 h, MG132 was added to 10 uM to the fourth sample, and all samples harvested at 24 h. Shown are blots for the FLAG tag, Bag6 and Ubl4A. The Bag6 and Ubl4A blots show that the exogenous proteins are expressed ~2X that of endogenous proteins. TRC35 could not be assessed similarly because the C-terminal antibody epitope is obscured by the FLAG tag, although we presume its levels are similar to Bag6-FLAG based on the FLAG blot. Note that ΔUbl-Bag6-FLAG is not detected by the Bag6 antibody, whose antigen includes the deleted domain, but its expression level was confirmed by the FLAG blot to be equal to Bag6-FLAG. This same ratio of Bag6 complex proteins was used in Fig. 4f, 4g, and Sup. Fig. S18. PrP expressed in these cells was of Hamster origin, and can be detected specifically using the 3F4 antibody. The endogenous Ubl4A serves as a convenient loading control in these experiments.



Sup. Fig. S21. Quantification of PrP stabilization by ΔUbl-Bag6 dominant negative expression. Mouse N2a cells in a 24-well dish were transfected with wild type PrP and either the Bag6 complex, ΔUbl-Bag6 complex, or control vectors, as in Sup. Fig. S20. The samples were harvested and immunoblotted to detect PrP (insets). The glycosylation pattern is a characteristically heterogeneous mixture of core and complex glycans, unlike the simpler in vitro translated product (e.g., Fig. 1) which only recieves core glycans. The graphs show a normalized densitometry scan down each lane, color coded as indicated. Note that ΔUbl-Bag6 complex expression leads to a ~2-fold selective increase in non-glycosylated PrP (left panel). The same level of increase is observed with 2 h proteasome inhibition on samples analyzed in parallel (right panel). Note also that there is a slight change in the glycoform pattern upon overexpression of either the Bag6 complex or Δ Ubl-Bag6 complex. This is probably due to indirect effects on vesicular trafficking secondary to slight differences in SNARE levels, given that Bag6 complex is involved in their insertion. Similar subtle changes in glycoforms were observed (in the opposite direction) upon shRNA-mediated silencing of Bag6 (see Sup. Fig. S22). Importantly however, these effects were not dependent on the Ubl domain of Bag6, consistent with this domain being dispensible for the chaperoning activity of Bag6 involved in its TA insertion functions. Finally, we have observed that even wild type Bag6 complex can have a dominant-negative effect if the ratio of its subunits are not roughly equal. This is seen to a slight degree in Fig. 4d, where the translocation-deficient PrP construct is stabilized partially by wild type Bag6 complex.



Sup. Fig. S22. Effect of Bag6 silencing on PrP. Hela cells were co-transfected with expression plasmids for PrP, CFP, and one of two different shRNAs against human Bag6. As controls, an irrelevant shRNA and the empty shRNA vector were used. After 103 h, the cells were harvested and analyzed by immunoblotting against the indicated proteins. Note that Bag6 (and the associated Ubl4A) were reduced ~50% by the shRNA treatment. This had minimal effect on CFP expression levels, but led to a selective stabilization of non-glycosylated PrP. The basis of the two bands for CFP is not clear, but may be due to alternative start codon usage or some degradation given that non-native residues are appended to CFP via translation through the polylinker of this empty plasmid. The non-glycosylated species is due to inefficient translocation since it is not observed upon increasing signal sequence efficiency (e.g., Fig. 4e). The slightly different glycosylation patterns between this and other experiments (e.g., Fig. 4e, Sup. Fig. S21) are due to cell type specific differences.



Sup. Fig. S23. Analysis of Sec61β ubiquitination. (a) Sec61β was translated in RRL supplemented with 10 uM His-Ub for 1 h. One reaction contained RMs, while another did not. The reaction containing RMs was assayed at various time points for membrane insertion using a protease-protection assay (Stefanovic and Hegde, 2007, Cell, 128:1147-59). The reaction lacking RMs was assayed at various time points for ubiquitination by pulldowns with immobilized Co^{+2}. The relative amounts (normalized to the 60 min value) of synthesis, insertion, and ubiquitination were quantified and plotted. Note that insertion is very closely timed with synthesis, while ubiquitination is slower and lags at least 10 min behind synthesis. (b) Sec61β was translated in RRL that was mock-depleted ('cont.'), TRC40-depleted, or TRC40-depleted and replenished to various levels with recombinant zebrafish TRC40. All reactions contained His-ubiquitin at 10 uM. The ubiquitinated products of each reaction were captured with immobilized Co^{+2} and analyzed. The amount of TRC40 in each reaction is indicated, relative to that in RRL.