Supporting Information

Leznicki and High 10.1073/pnas.1209997109

SI Materials and Methods

Recombinant human FLAG-tagged ubiquitin, methylated ubiquitin, and ubiquitin aldehyde were from R&D Systems and HA-tagged ubiquitin vinyl sulfone was from Enzo Life Sciences. Rabbit reticulocyte lysate used for ubiquitination and in vitro translation was from Promega. Antibodies recognizing FLAG (Sigma), V5 (Serotec), GFP (Roche), ubiquitin and the 20S proteasome (Enzo Life Sciences), Vpu (Acris GmbH), and SGTA (Santa Cruz Biotechnology) were purchased. Anti-opsin and chicken anti-BAG6 are as described (1), and rabbit anti-C99, BAG6, and Sec61β were made to order.

Protein Expression and Recombinant Proteins. SGTA or its deletion mutants (Fig. 4*C*) were subcloned into pcDNA5/FRT/V5-His (Invitrogen) to incorporate the C-terminal V5-His tag. APP-C99 was subcloned into the same vector without the C-terminal tag, and PPL-C99 bearing the wild-type signal sequence of bovine preprolactin was in pcDNA3.1 (2). Recombinant tail-anchored proteins were expressed and purified as previously described (1). HisTrx-SGTA, HisTrx, His-VSV-G-TRC40, and His-S-SGTA or its deletion mutants (Fig. 2*F*) were expressed as described for tail-anchored proteins (1), bacteria resuspended in buffer A [50 mM Hepes·KOH (pH 7.5), 300 mM NaCl, 10 mM imidazole, 10% (vol/vol) glycerol, 5 mM 2-ME] supplemented with 1 mM PMSF and complete protease inhibitor mixture (Roche), and lysed by sonication on ice (3 × 30-s pulses with 1-min intervals). Insoluble material was removed by centrifugation, 10,000 × *g* for 30 min and the soluble fraction incubated for 3 h at 4 °C with preequilibrated NiNTA Agarose beads (Qiagen). Bound material was washed extensively with buffer A; buffer A supplemented with 1% (vol/vol) Triton X-100, 1 M NaCl, 5 mM ATP and 5 mM $MgCl_2$; and then with buffer A containing 0.5 M Tris·Cl (pH 7.4). Resin was washed again with buffer A and proteins eluted in a stepwise manner with increasing concentrations of imidazole in buffer A. Fractions of highest protein content were desalted, buffer exchanged to PBS, and protein concentration estimated by densitometry using BSA or lysozyme as a standard.

1. Leznicki P, Clancy A, Schwappach B, High S (2010) Bat3 promotes the membrane integration of tail-anchored proteins. *J Cell Sci* 123(Pt 13):2170–2178.

2. Wilson CM, et al. (2005) Ribophorin I associates with a subset of membrane proteins after their integration at the sec61 translocon. *J Biol Chem* 280(6):4195–4206.

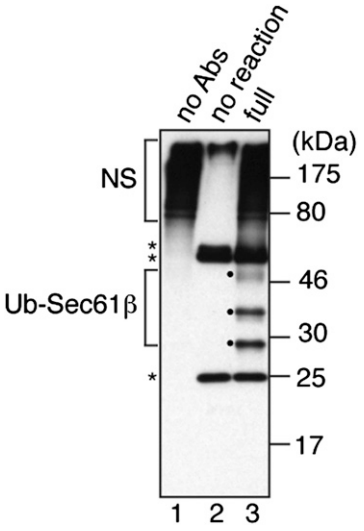
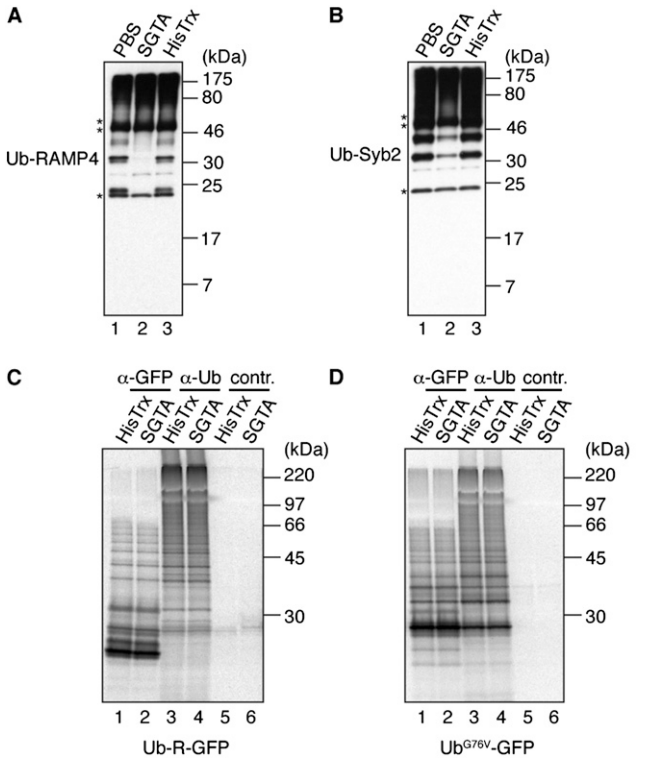


Fig. S1. Defining specific Sec61β-ubiquitinated species. Purified recombinant Sec61β (2 μM) was mixed with rabbit reticulocyte lysate supplemented with 40 μM FLAG-tagged ubiquitin and incubated at 30 °C for 2 h then treated with 5 mM *N*-ethylmaleimide (NEM). Samples diluted in immunoprecipitation buffer were then incubated with Protein A Sepharose alone (lane 1) or an antibody recognizing the opsin-derived epitope tag present in recombinant Sec61β and then Protein A Sepharose (lane 3). Alternatively, just the anti-opsin antibody and Protein A Sepharose were incubated in the absence of any other components (lane 2). Protein A-bound material was resolved by SDS/PAGE and anti-FLAG antibody-reactive species were identified by immunoblotting. Specific ubiquitinated Sec61β species were only observed in the complete reaction (lane 3, ●). Anti-FLAG–reactive, high-molecular-weight species bound nonspecifically to Protein A Sepharose (NS) in the absence of the anti-opsin antibodies (lane 1), whereas the heavy (**) and light (*) chains of the anti-opsin antibody cross-react with the secondary antibody used to detect anti-FLAG (lane 2).



E

substrate	Ub_{HisTrx}/Ub_{SGTA}
OP91	2.62
Vpu	1.86
APP-C99	2.11
Ub-R-GFP	1.01
Ub^{G76V}-GFP	1.16

Fig. S2. SGTA-mediated inhibition of ubiquitination is substrate-specific. Recombinant RAMP4 (*A*) or Synaptobrevin 2 (Syb2) (*B*) were ubiquitinated in vitro in the presence of SGTA, HisTrx, or PBS as described in the legend to Fig. 2*A*. Cross-reaction with antibody heavy (**) and light (*) chains is indicated as before. Ub-R-GFP (*C*) and Ub^{G76V}-GFP (*D*) were translated in rabbit reticulocyte lysate in the presence of 2 μM HisTrx-SGTA (SGTA) or HisTrx control, treated with 5 mM NEM and immunoprecipitated with antibodies against GFP, ubiquitin, or a control (contr.). Immunoprecipitated material was resolved by SDS/PAGE and visualized by phosphorimaging. (*E*) For each precursor indicated, the amount of ubiquitinated species recovered by immunoprecipitation following cell free translation in the absence and presence of SGTA was determined by phosphorimaging analysis of the data shown in Fig. 2 *C–E* and Fig. S2 *C* and *D*. The resulting values were expressed as a ratio of ubiquitinated material in the control to ubiquitinated material in the presence of SGTA for each precursor as indicated.

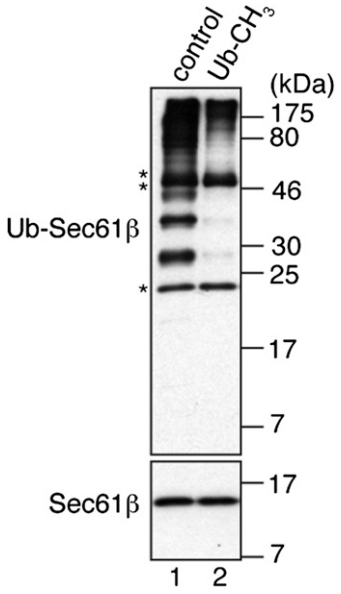


Fig. S3. Methylated ubiquitin inhibits Sec61β ubiquitination. Sec61β was incubated for 2 h at 30 °C in proteasome-depleted rabbit reticulocyte lysate supplemented with 40 μM FLAG-tagged ubiquitin plus either buffer (control) or 120 μM methylated ubiquitin (Ub-CH_3). Reactions were stopped by NEM treatment, Sec61β immunoprecipitated as before, and samples analyzed by Western blotting with anti-FLAG and anti-Sec61β antibodies.

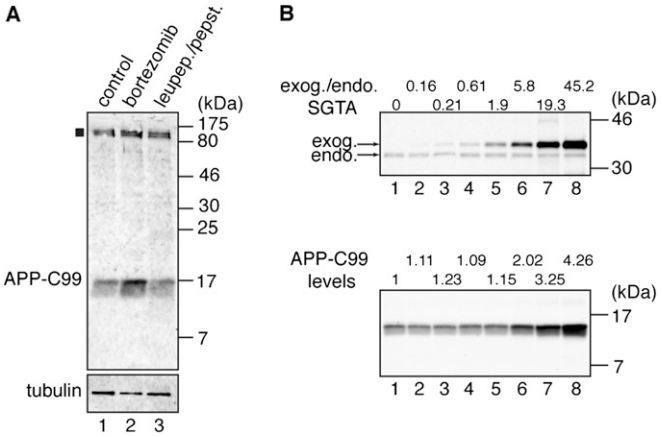


Fig. S4. APP-C99 is proteasomal substrate that is stabilized by SGTA coexpression. (*A*) HeLa cells were cotransfected with a plasmid encoding APP-C99 plus empty vector and treated with DMSO (control), bortezomib, or leupeptin/pepstatin (leupep./pepst.) overnight as indicated. Substrate levels were examined 22 h posttransfection by quantitative Western blotting of total cell lysate. ■, Cross-reacting species observed with the anti-C99 antibody. (*B*) HeLa cells were cotransfected with pcDNA5-APP-C99 and a mixture of empty pcDNA3.1 plus pcDNA5-SGTA-V5 at increasing ratios of the latter. SGTA (*Upper*) and substrate (*Lower*) levels were examined 22 h posttransfection by quantitative Western blotting of total cell lysate. The migration of endogenous (endo.) and exogenous (exog.) SGTA is indicated, together with the degree of SGTA overexpression relative to the endogenous protein obtained with increasing amounts of plasmid (exog./endo.). Levels of APP-C99 were normalized to endogenous SGTA and are expressed relative to the amount of substrate recovered in cells not expressing exogenous SGTA (*Lower*, lane 1).