Expanded View Figures

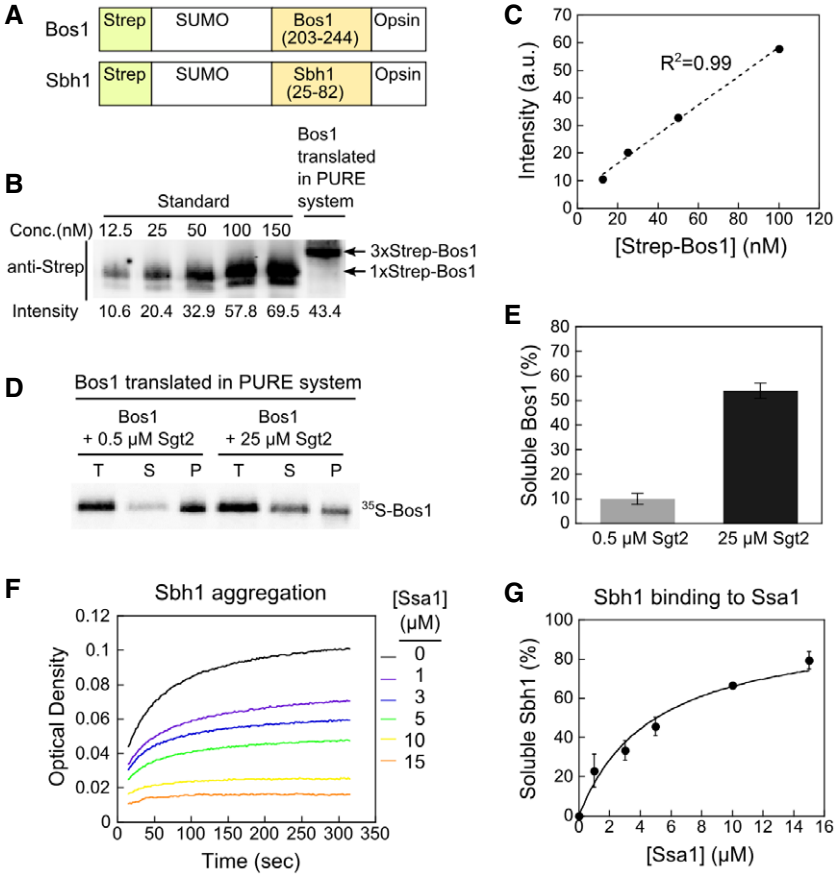

Figure EV1. Sgt2 prevents TA aggregation only at super-physiological concentrations.

A Schematic of the model TA substrates used in *in vitro* assays. A Strep-tagged non-cleavable SUMO protein was fused to Bos1 residues 203–244 or Sbh1 residues 25–82 that encompasses their targeting sequence, which includes an N-terminal linker to the cytosolic domain, the TMD, and the C-terminal sequence element that regulates insertion into the ER (Rao *et al*, 2016). An opsin tag was attached to C-terminus of model TAs. For IVT, a 3xStrep tag was fused to SUMO in Bos1 instead of 1xStrep tag.

B, C Quantitative Western blot analysis of Bos1 synthesized in the PURE-IVT system. Known amounts of purified Strep-Bos1 were used to construct a standard curve (panel B) for quantification of the concentration of 3xStrep-Bos1 translated in the PURE system. Proteins were detected with a secondary antibody labeled with a near-infrared fluorescent dye, IRDye. The 3xStrep-Bos1 synthesized in PURE-IVT was 71 nM, which is sub-stoichiometric to the chaperones used in this work.

D, E Sedimentation analysis of ^{35}S-labeled Bos1 synthesized by PURE-IVT supplemented with 0.5 μM or 25 μM Sgt2. After ultracentrifugation, total input (T), soluble (S), and pellet (P) fractions were resolved by SDS–PAGE and visualized by autoradiography. The quantification of soluble Bos1 at indicated Sgt2 concentrations is shown in (E). All values are mean ± SD, with $n \geq 3$.

F, G Ssa1 suppresses Sbh1 aggregation in a dose-dependent manner in the turbidity assay. Panel (G) shows the quantification of the data in (F) and replicates. The line was a fit of the data to equation (1) and gave a $K_{soluble}$ value of 4.8 ± 0.9 μM. All values are reported as mean ± SD, with $n = 2$.

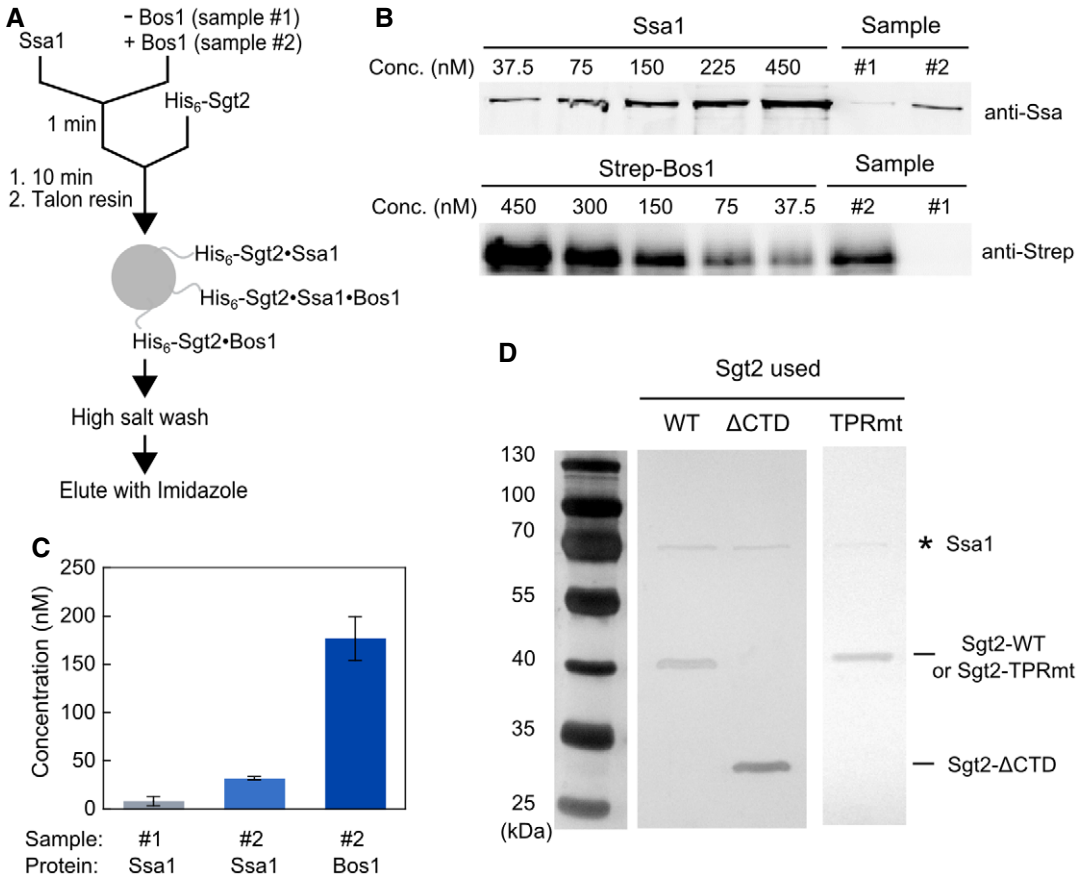

Figure EV2. Ssa1 that co-purified with Sgt2 in the His_6-pull-down assay accounted for < 17% of Sgt2-associated TA.

A Scheme of the His_6-Sgt2 pull-down assay to monitor TA loading onto Sgt2 used for the experiments in Fig 4C. 5 μM Ssa1 and 2 mM ATP were pre-incubated in the absence (sample #1) or presence (sample #2) of 300 nM Strep-Bos1 for 1 min, and further incubated for 10 min after addition of 500 nM His_6-Sgt2. The mixture was incubated with Talon resin at 4°C for 10 min. All three possible complexes that can be immobilized on the resin via His_6-Sgt2 are depicted. After washing the resin with high-salt (500 mM NaCl) buffer, bound proteins were eluted with 300 mM imidazole.

B Quantitative Western blot analysis of the amount of Ssa1 and Bos1 in the elution fractions from samples #1 and #2 in (A), using known concentrations of purified Ssa1 and strep-Bos1. Proteins were detected with their corresponding secondary antibodies labeled with IRDye.

C Quantified concentrations of Ssa1 and Bos1 that co-purified with Sgt2 in the elution fractions: 8.2 nM Ssa1 in the absence of Bos1 (sample #1), and 32 nM Ssa1 and 180 nM Bos1 in the presence of Bos1 (sample #2). Values are reported as mean ± SD, with $n \geq 2$.

D Silver-stained images of Sgt2·TA complexes reconstituted and purified as in panel (A) for wild-type and mutant Sgt2. * denotes the Ssa1 band. Note that this gel was overdeveloped to visualize the presence of Ssa1.

Source data are available online for this figure.

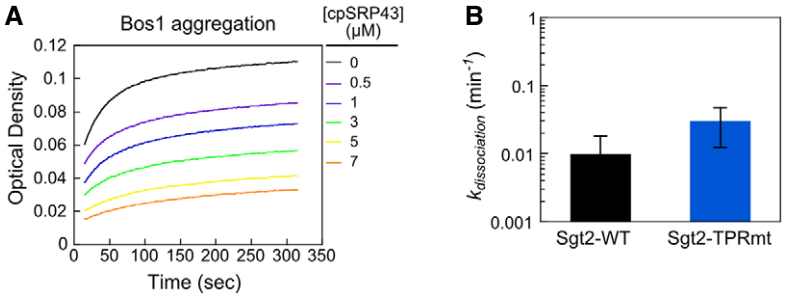

Figure EV3. cpSRP43 can serve as a trap for TA proteins.

A cpSRP43 prevents Bos1 aggregation in a dose-dependent manner, as analyzed by the turbidity assay.

B The intrinsic dissociation rate constants of Bos1 from the Sgt2-WT·Bos1 and Sgt2-TPRmt·Bos1 complexes reconstituted via IVT in *E. coli* extract. $Bos1^{CM}$ was translated in the *E. coli* S30 translation extract coupled to amber suppression, which co-translationally incorporates the non-natural fluorescent amino acid L-(7-hydroxycoumarin-4-yl)ethylglycine (CM) into Bos1 near the TMD, in the presence of 2 μM His_6-$Sgt2\text{-}WT^{BFL}$ or His_6-$Sgt2\text{-}TPRmt^{BFL}$, as described in (Rao *et al*, 2016). The resulting His_6-$Sgt2\text{-}WT^{BFL}$·$Bos1^{CM}$ or His_6-$Sgt2\text{-}TPRmt^{BFL}$·$Bos1^{CM}$ complexes were purified using metal affinity resin (Rao *et al*, 2016). $Bos1^{CM}$ release from $Sgt2\text{-}WT^{BFL}$ or $Sgt2\text{-}TPRmt^{BFL}$ was measured as outlined in Fig 4E. Values are mean ± SEM, with $n = 2$.

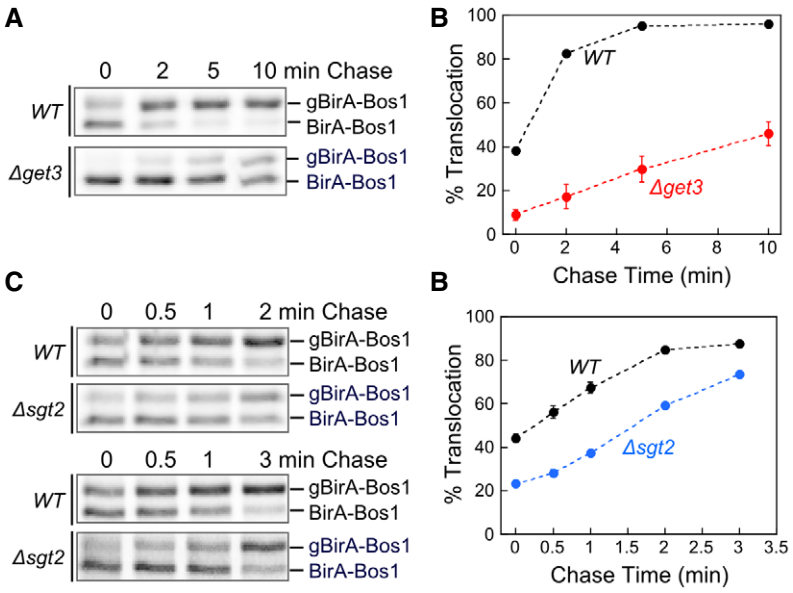

Figure EV4. BirA-Bos1 was inserted into the ER membrane in a GET-dependent manner.

A Representative autoradiograms for pulse-chase analysis of the translocation of metabolically labeled BirA-Bos1 in *WT* (black) and Δ*get3* (red) cells.

B Quantification of the data in (A) and replicates.

C Representative autoradiograms for pulse-chase analysis of the translocation of metabolically labeled BirA-Bos1 in *WT* (black) and Δ*sgt2* (blue) cells.

D Quantification of the data in (C) and replicates.

Data information: Values in (B) and (D) are mean ± SD, with $n = 2$ (biological replicates). Error bars are shown but may not be visible in some cases.

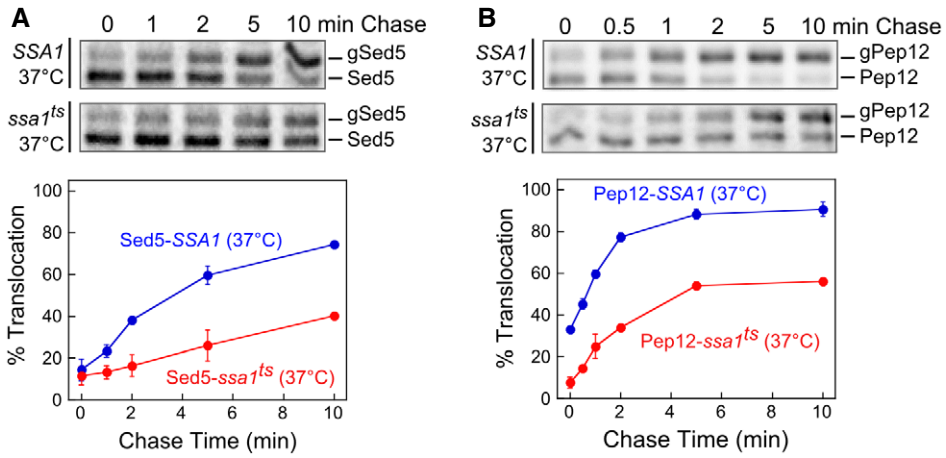

Figure EV5. Ssa1 facilitates the targeting of multiple GET-dependent TAs to the ER.

A, B The top panels show representative autoradiograms for pulse-chase analysis of the translocation of metabolically labeled Sed5 (A) and Pep12 (B) in *SSA1* (blue) and *ssa1*^{ts} (red) cells at 37°C. The bottom panels show the quantifications of translocation efficiencies at indicated time points. All values are mean ± SD, with n = 2 (biological replicates). Error bars are shown but may not be visible in some cases.