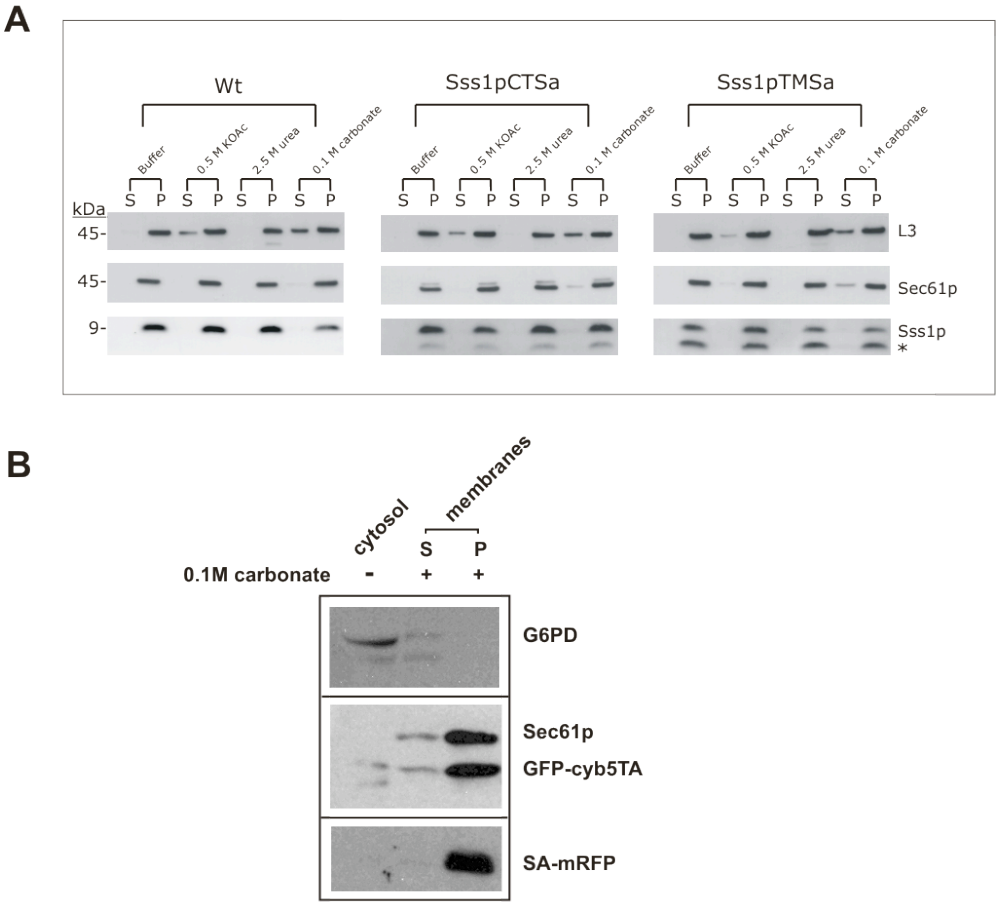
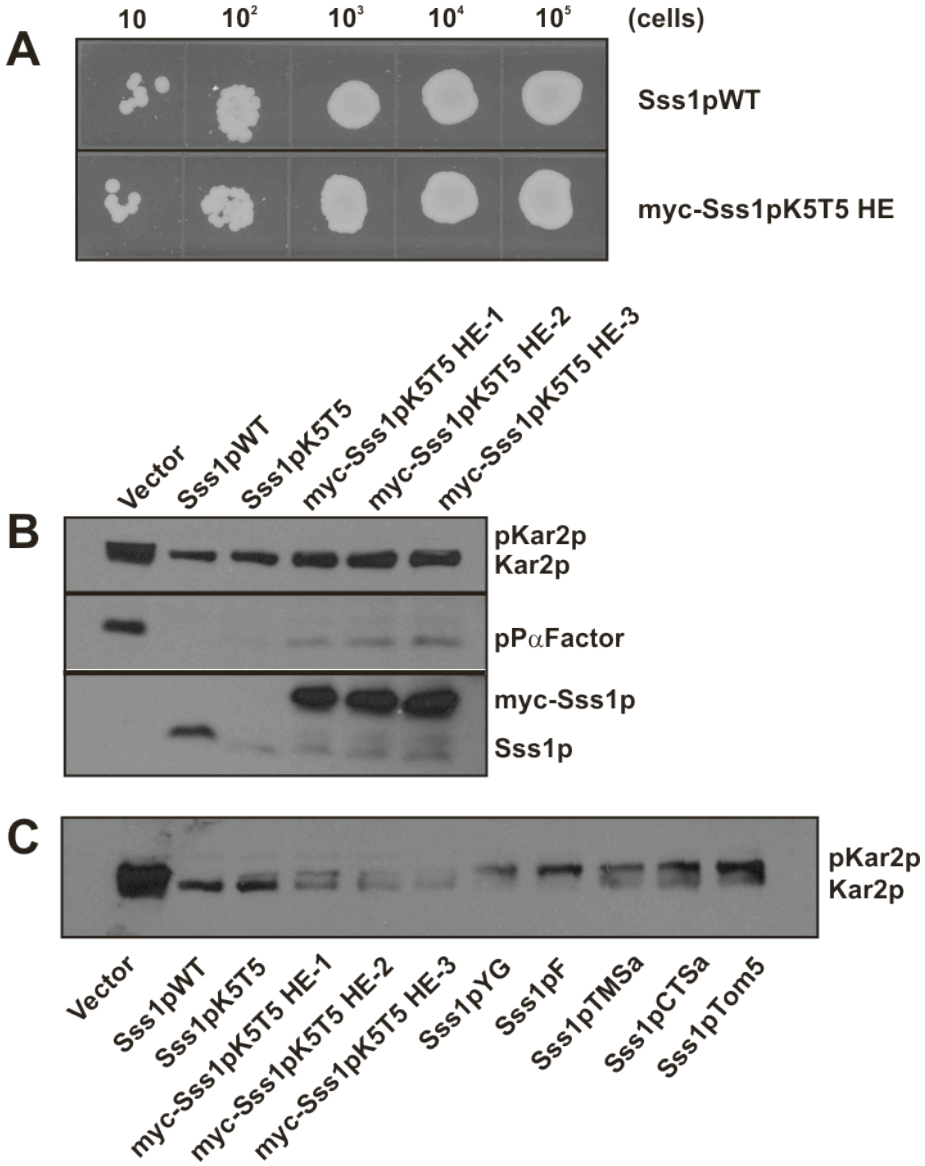
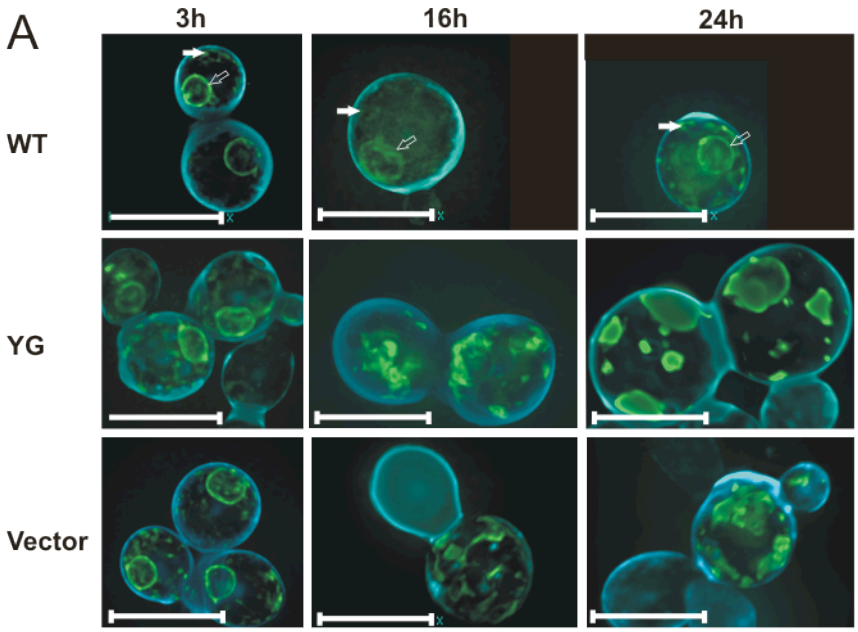


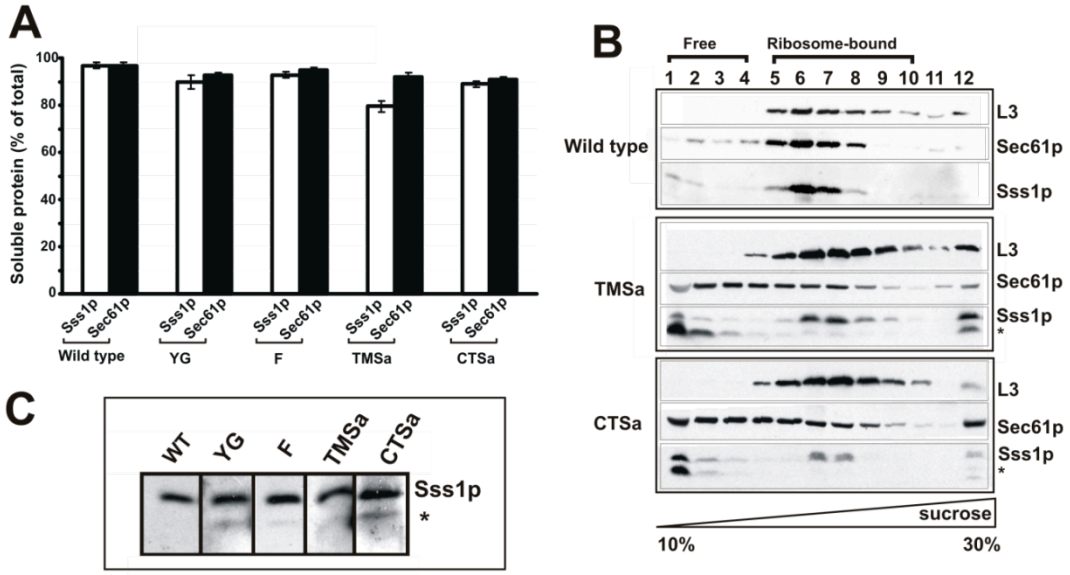
Supplemental Figure 1. *Sss1p mutants and GFP-Cyb5TA and SA-mRFP reporters are integral membrane proteins.* (A) Purified rough microsomes (Meisinger C, Sommer T and Pfanner N. Anal Biochem. 2000 Dec 15;287(2):339-42.) from FKY173 strains expressing Sss1pWT, Sss1pCTSa, and Sss1pTMSa were treated with buffer as a control, 0.5M potassium acetate, 2.5M urea or 0.1M sodium carbonate, pH 11.5. Membranes were pelleted at 100 000*xg* to generate a supernatant (S) and pellet (P) fraction. Fractions were separated by SDS-PAGE, transferred to nitrocellulose and proteins were analyzed by immunodetection using antibodies directed against Sec61p and the large ribosomal subunit L3. *A cleavage product of Sss1p, which co-fractionates with the full-length protein. (B) Post-nuclear membranes from FKY173 strain expressing GFP-Cyb5TA and SA-mRFP were separated from cytosol, treated with 0.1M sodium carbonate, pH 11.5 and processed as described in (A). Blots were decorated with antibodies against cytosolic marker glucose-6-phosphate dehydrogenase (G6PD), ER marker Sec61p, GFP and mRFP.



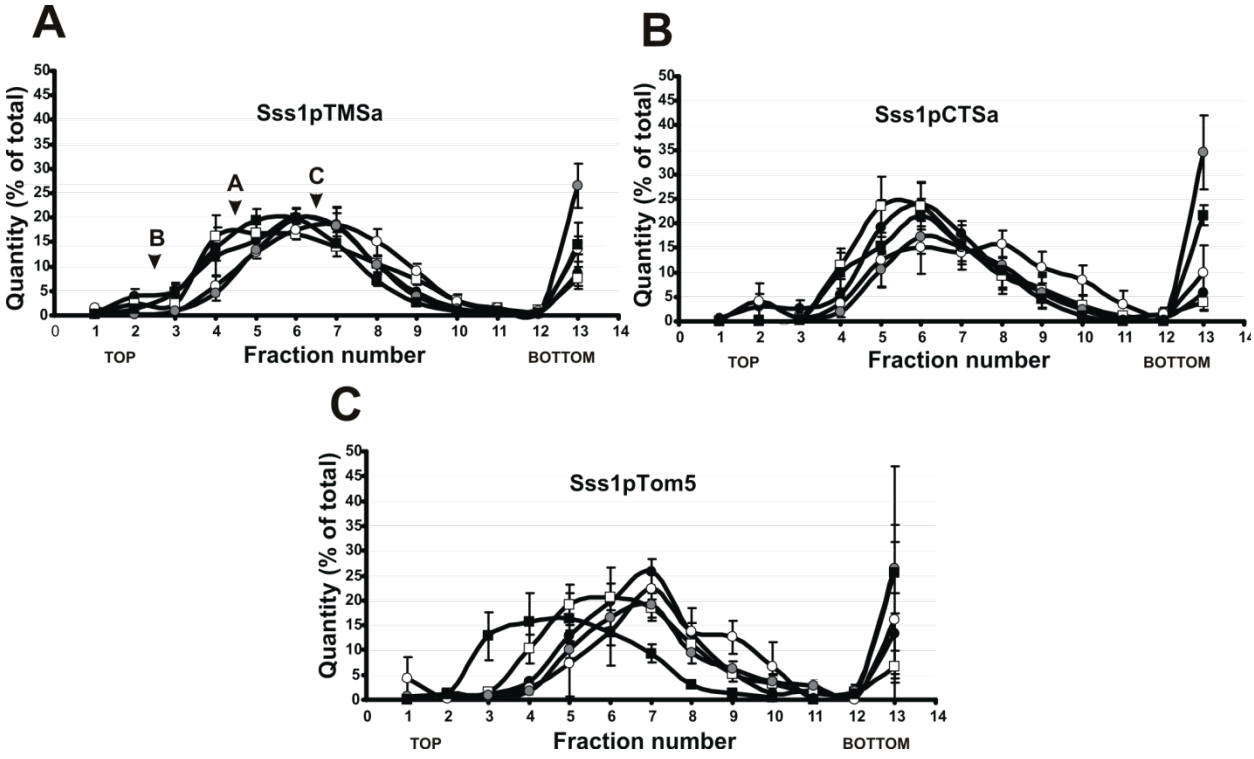
Supplemental Figure 2. *Over expression of Sss1pK5T5 does not result in growth or extreme translocation defects.* (A) 10-fold serial dilutions of exponentially growing cultures of the FKY173 strain carrying a low copy plasmid expressing wild-type Sss1p and carrying a high copy plasmid expressing a myc-tagged K5T5 mutant, were spotted onto solid minimal media plates (minus adenine and histidine) supplemented with 0.2% amino acids and 2% glucose, and incubated at 30˚C for 3 days. (B) Expression levels of Sss1p and myc-Sss1p, and accumulation of pre-Kar2p and prepro-alpha-factor in yeast strain FKY173 transformed with an empty plasmid (vector) or with plasmids expressing mutant Sss1p proteins. Myc-Sss1pK5T5HE indicates a strain expressing the myc-tagged K5T5 mutant in a high expression vector; numbering indicates samples from 3 individual colonies. Immunoblot analysis was performed on whole cell lysates of yeast cells grown for 8 hours at 30°C. (C) Accumulation of pre-Kar2p for all Sss1p mutants including lysates from 3 individual colonies expressing the myc-tagged K5T5 in a high expression vector.



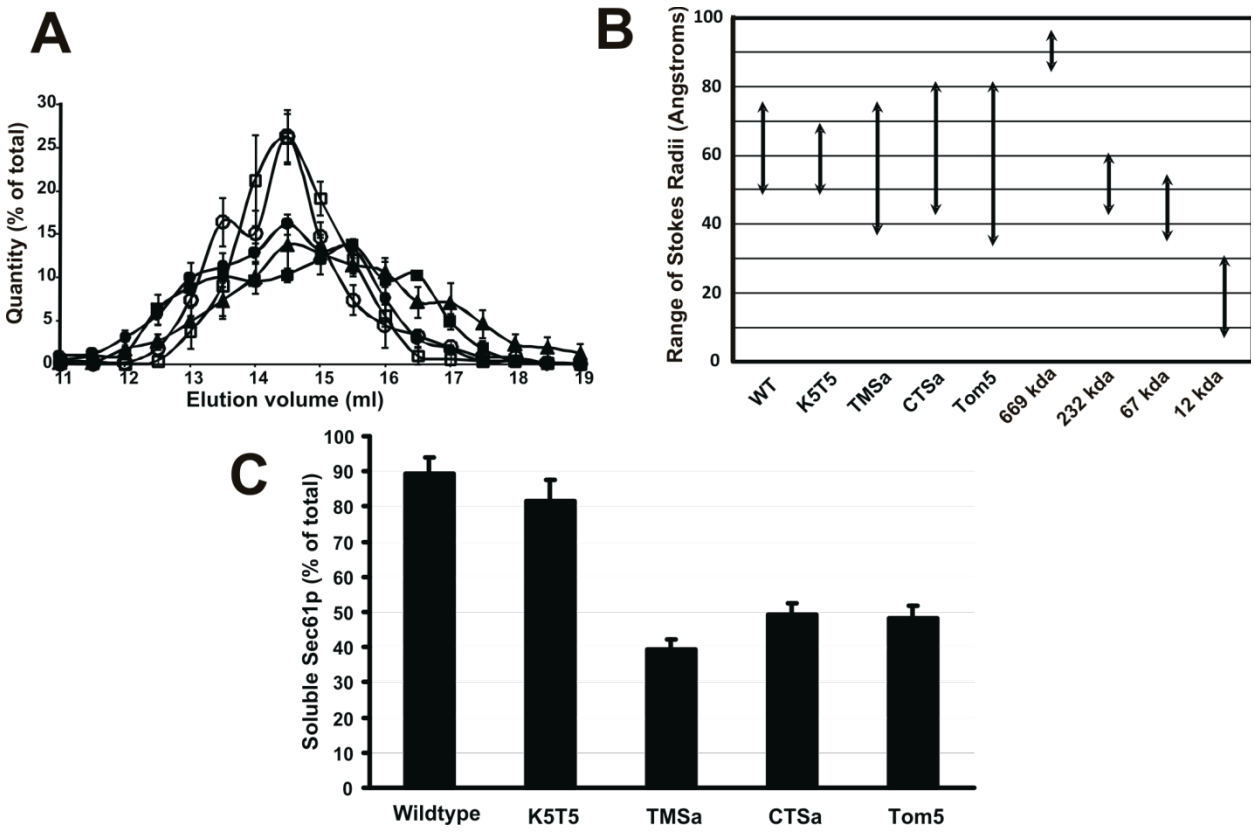
Supplemental Figure 3. *Depletion of wild-type Sss1p and expression of Sss1pYG result in changes to the structure of the ER.* Deconvolved wide-field microscopic images of yeast strain FKY173 showing expression of GFP-Cyb5TA at 3, 16 and 24 hours after growth in glucose to suppress expression of wild-type Sss1p. Solid arrows indicate normal peripheral ER; open arrows indicate normal peri-nuclear ER. The cell wall is stained cyan with Calcofluor White. Scale bar: 7μm.



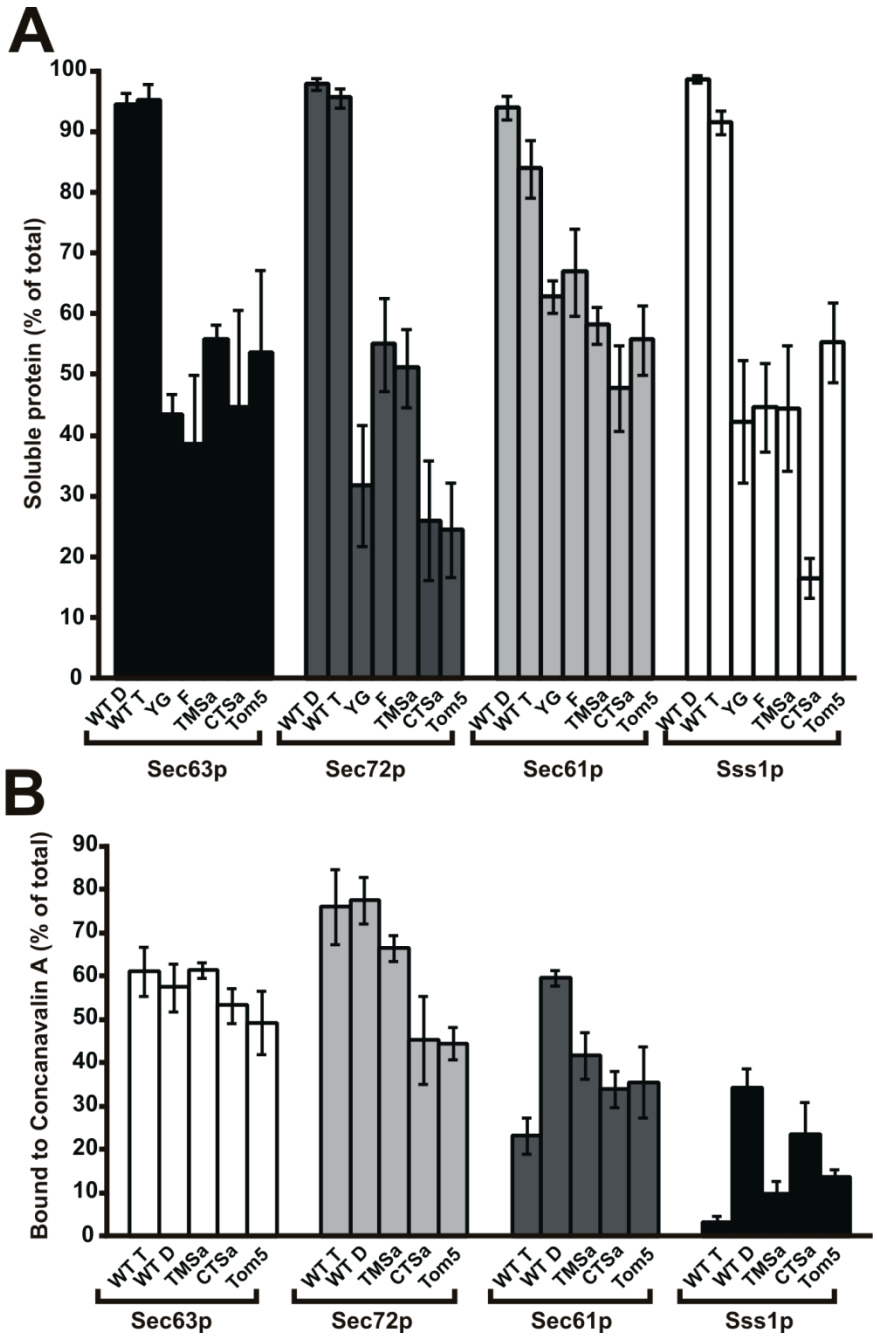
Supplemental Figure 4. (A) Ribosome –Sec 61 complexes separated by sucrose gradient centrifugation in low ionic strength buffer are soluble. Samples prepared as described in Figure 3 were quantified by expressing the total amount of Sec61p and Sss1p in the soluble fractions (fractions 1-11) as a percent of the total in all fractions. (B) Defects in ribosome interactions with Sec61p complexes prepared (as described in Figure 3) from yeast expressing Sss1pTMSa and Sss1pCTSa. * denotes a degradation product of Sss1p. (C) Degradation of Sss1p mutants is minimal at the time of solubilization. Samples from strains expressing wild-type, YG, F, TMSa, and CTSa Sss1p proteins were prepared as in A and separated by SDS-PAGE prior to sucrose gradient centrifugation. Immunoblots were probed for Sss1p.



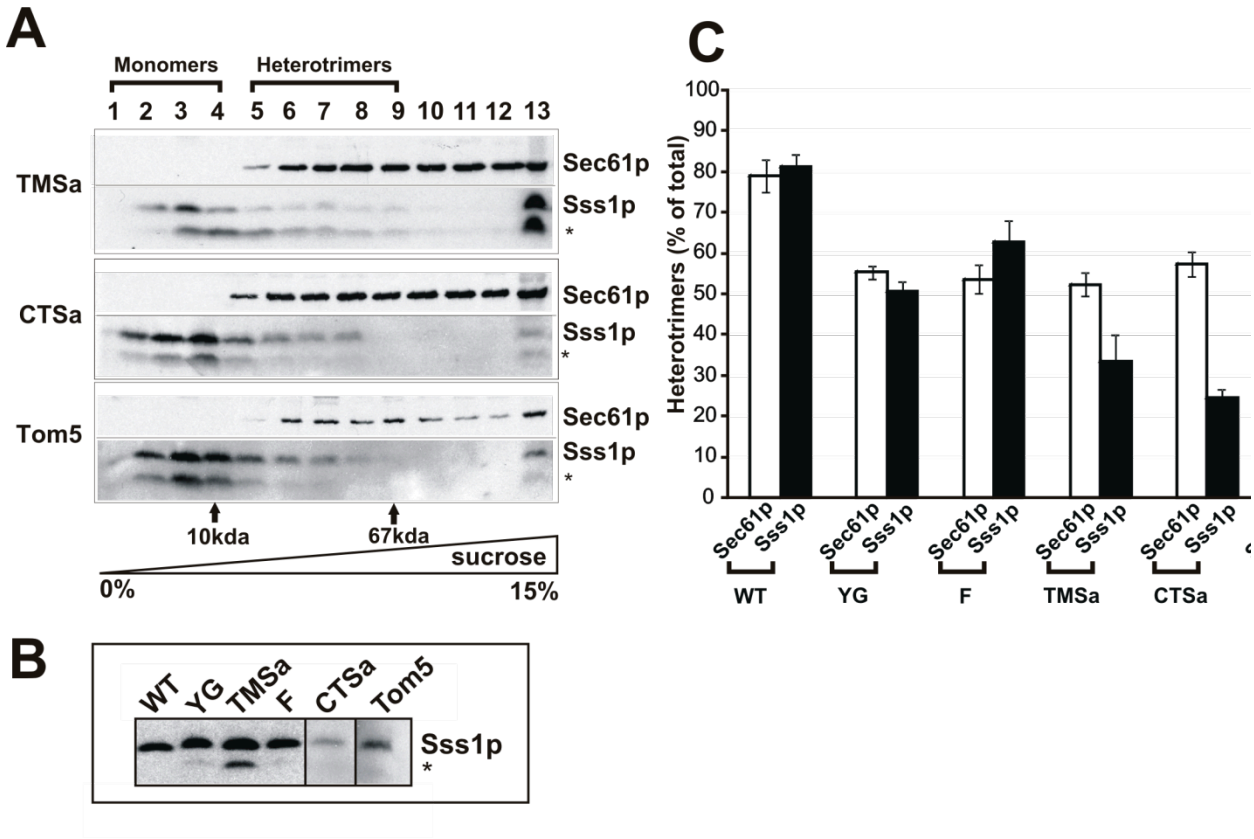
Supplemental Figure 5. *Digitonin-soluble Sec complexes remain intact but altered in membranes prepared from yeast strains expressing TMSa, CTSa, and Tom5 mutants.* Post-nuclear membranes derived from yeast expressing Sss1pTMSa (A), Sss1pCTSa (B), or Sss1pTom5 (C) were solubilized in 2.5% Digitonin buffer and complexes were resolved by sucrose gradient centrifugation in the same buffer (except the concentration of detergent was reduced to 0.1%) containing a linear gradient of 25-35% sucrose. Gradients were divided into 13 fractions, precipitated, and analyzed by SDS-PAGE. Immunoblots were probed with antibodies to Sec63p (black circles), Sec62p (white circles), Sec72p (grey circles), Sec61p (white squares), and Sss1p (black squares). Bands in each fraction were quantified, expressed as a percent of the total, and plotted by fraction number to generate a sedimentation profile. Positions of molecular weight standards are marked: B, BSA (67 kda), A, Aldolase (158 kda), C, Catalase (232 kda).



Supplemental Figure 6. *Digitonin-soluble Sec complexes separated by gel filtration remain intact but altered in membranes prepared from yeast strains expressing tail-anchor mutants of Sss1p.* (A) Post-nuclear membranes derived from yeast expressing Sss1pWT (white circles), Sss1pK5T5 (white squares), Sss1pTMSa (black triangles), Sss1pCTSa (black circles), and Sss1pTom5 (black squares) were salt extracted, solubilized in 2.5% Digitonin buffer and Sec complexes were resolved by separation on a Superose 6 HR 10/30 FPLC column and run as described in the *Materials and Methods* for the Superdex 200 column. Samples eluting from 11 to 19 ml were collected in 0.5ml fractions, precipitated, and resolved by SDS-PAGE. Immunoblots were probed with antibodies to Sss1p, quantified, expressed as a percent of the total, and plotted by volume to generate an elution profile. (B) The range of Stokes' radii at 50% of the peak height was calculated for each mutant from the data in (A) and plotted. Stokes' radii of molecular weight standards are shown for reference: Thyroglobulin, 669 kda; Catalase, 232 kda; BSA, 67 kda; Cytochrome c, 12 kda. (C) Solubility of Sec61p in Sec complexes derived from yeast expressing wild-type and mutant Sss1p proteins. Post-nuclear membranes were prepared as described in (A). Solubility was calculated by quantifying the amount of Sec61p in the pre-cleared supernatant and expressing it as a percent of the total of the pellet and supernatant fractions.



Supplemental Figure 7. (A) Solubility of Sec complex proteins in Concanavalin A binding assay. Solubilized samples were prepared as described in Figure 5 and bands present in the supernatant were quantified and expressed as a percent of the sum of supernatant and pellet. (B) Immunoblots from **e**xperiments performed as described in Figure 5 were quantified and expressed as percent bound to Concanavalin A. WT T, wild-type Sss1p membranes solubilized in 1% Triton X-100 Buffer; WT D, TMSa D, CTSa D, Tom5 D, digitonin-solubilized membranes from strains expressing Sss1pWT, Sss1pTMSa, Sss1pCTSa , and Sss1pTom5, respectively.



Supplemental Figure 8. *TMSa, CTSa, and Tom5 mutants of Sss1p form unstable heterotrimeric Sec61p complexes.* (A) Post-nuclear membranes derived from yeast strains expressing Sss1pTMSa, Sss1pCTSa, or Sss1pTom5 were prepared as described for Figure 6, separated by SDS-PAGE and blots were decorated with antibodies to Sec61p and Sss1p. The positions of molecular weight standards run in parallel experiments are shown: Cytochrome c, 12 kda; BSA, 67 kda. (B) Degradation of Sss1p mutants is minimal at the time of solubilization. Samples from strains expressing wild-type, YG, TMSa, F, CTSa and Tom5 Sss1p proteins were prepared as described in (A) and separated by SDS-PAGE prior to sucrose gradient centrifugation. Immunoblots were probed for Sss1p. * denotes the degradation product of Sss1p. (C) Immunoblots from samples prepared as described in (A) for all Sss1p strains were quantified and the amount of Sss1p and Sec61p sedimenting as heterotrimers (fractions 5-9, inclusive) was expressed as a percent of the total in all 13 fractions.

A

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Homo sapiens              NP_001012474.1       1   ------------------MDQVMQFVEPSRQFVKDSIRLVKRCTKPDRK   31
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Canis lupis familiaris    NP_001003325.1       1   ------------------MDQVMQFVEPSRQFVKDSIRLVKRCTKPDRK   31
Bos Taurus                NP_001035676.1       1   ------------------MDQVMQFVEPSRQFVKDSIRLVKRCTKPDRK   31
Mus musculus              NP_035473.1          1   ------------------MDQVMQFVEPSRQFVKDSIRLVKRCTKPDRK   31
Rattus norvegicus         XP_001069684.1      51   RYWRCERPAGRLGPGRGTNMDQVMQFVEPSRQFVKDSIRLVKRCTKPDRK   100
Gallus gallus             XP_001236152.1       1   ------------------MDQVMQFVEPSRQFVKDSIRLVKRCTKPDRK   31
Drosophila malanogaster   NP_608337.1          1   ------------------MDKVVKFAEPGRAFAKDSIRLVKRCTKPDRK   31
Caenorhabditis elegans    NP_505778.1          1   ------------------MDQFQALIEPARQFSKDSYRLVKRCTKPDRK   31
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Oryza sativa              NP_001058242.1       1   ------------------MDAIDSVVDPLREFAKDSVRLVKRCHKPDRK   31
Saccharomyces cerevisiae  NP_010371                  MARASEKGEEKKKQSNNQVEKLVEAPVEFVREGTQFLAKCKKPDLK   46

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Pan troglodytes           XP_001152525.1      32   EFQKIAMATAIGFAIMGFIGFFVKLIHIPINNIIVGG-   68
Canis lupis familiaris    NP_001003325.1      32   EFQKIAMATAIGFAIMGFIGFFVKLIHIPINNIIVGG-   68
Bos Taurus                NP_001035676.1      32   EFQKIAMATAIGFAIMGFIGFFVKLIHIPINNIIVGG-   68
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Gallus gallus             XP_001236152.1      32   EFQKIAMATAIGFAIMGFIGFFVKLIHIPINNIIVGG-   68
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Caenorhabditis elegans    NP_505778.1         32   EYQKIAMATAIGFAIMGFIGFFVKLIHIPINNIIVGA-   68
Arabidopsis thaliana      NP_568728.1         32   EFTKVAVRTAIGFVVMGFVGFFVKLIFIPINNIIVGAT   69
Oryza sativa              NP_001058242.1      32   EFSKVALRTAIGFVVMGFVGFFVKLIFIPINNIIVGSG   69
Saccharomyces cerevisiae  NP_010371          47   EYTKIVKAVGIGFIAVGIIGYAIKLIHIPIRYVIV---   81
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B

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                          10        20        30        40        50        60        70
                   ....*....|....*....|....*....|....*....|....*....|....*....|....*....|....
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gi 14548254    1 MDISGMQINEVQSKLK---------EYYNVLKMARKPDWEEFSMTAKVALAVMFIVGFVGFVIYILMEILPGAL 65
gi 14916700    1 ----MGIVDIAREYIV---------AWRRILTLARKPDEEEYSLLLKLNLLGFALVGGIGYLIHLGYIILTSG- 60
gi 19173628    1 MSQKLQKPSFLSEYLR---------SIRLFSKKCVRPSGKELSMSIKRHAIGIGFLGILGYAIKLIHIPINNII 65
gi 22096152    9 NITTKSVGQAIR-------------AHLRVLKLTKKPSREEFLTIAKVAGAGILAVGAIGFIIYVLLTMLPQWV 69
gi 2495938     2 KTDFNQKIEQLKEFIE---------ECRRVWLVLKKPTKDEYLAVAKVTALGISLLGIIGYIIHVPATYIKGIL 66
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gi 14548257    1 ---MPEFQERVRNFFK---------ESKRVFLVTKKPGWEEYKKAAKITGLGILVIGLVGMLIRIIGILMLGG- 61
gi 1351034     3 DN-ADDLFQIPKNFYK---------EGSHFIKRCVKPDRKEFLSISKAVATGFVLMGLIGYIIKLIHIPINKVL 66
gi 16082624   31 LVSIEDKAEEIQQNLErrfssigkgKYARILRMARKPTRDEYIKVLVITAVGLLIIGAVGFIIYLLMDVYFKIP 104
gi 14548260    1 ----MDVPLELS-------------AYTRVLRLASTPSWEEFSQIAKIAGAGILLIGAIGFLVFLIMGGIVSVI 57
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gi 6320291    15 NNQVEKLVEAPVEFVR---------EGTQFLAKCKKPDLKEYTKIVKAVGIGFIAVGIIGYAIKLIHIPIRYVI 79 S. cerevisiae
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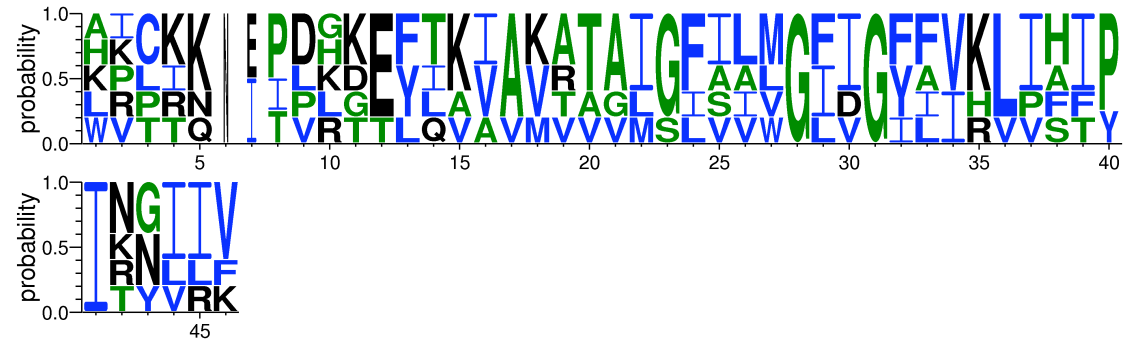
C



Supplemental Figure 9. *Sec61- γ is highly conserved.* (A) Shown are sequence alignments of Sec61γ from various species. Sequence identity is shown in red; the TMS is shown highlighted

in grey. (B) Sequence comparisons between Archaea and *S. cerevisiae* show that the two C-terminal glycines in the tail anchor are absolutely conserved. The sequence of SecE from *E. coli* K12 (sub strain W3110) is shown for comparison. The positions of four known prlG mutations are highlighted in green. The TMS of Sss1p is shown in grey. (C) Alignments of the tail-anchor region (NTS, TMS, & CTS) of Sec61γ (top to bottom) from *H. sapiens*, *A. thaliana*, *S. cerevisiae,* M. *jannaschii* and *E. coli* K12 (sub-strain W3110) using the Web Logo program (Crooks GE *et al*., 2004; Genome Research). Amino acid letter height represents the probability of that amino acid occurring at that position. Lines at position 6 on the x-axis represent regions that do not align.

Additional Supplemental Materials:

Plasmid construction details

To construct the WT Sss1p yeast expression vector, primers containing SalI and XbaI restriction sites were used to amplify the WT *SSS1* coding sequence, along with the endogenous promoter and 3'UTR, from purified yeast genomic DNA. The PCR product was then cloned as a SalI-XbaI fragment into pRS313.

To construct the K5T5 mutant, the five lysine residues in the N-terminal sequence of WT Sss1p were mutated to threonine residues by a mutagenic cassette inserted into the AflII and MfeI sites which had been created by whole plasmid PCR amplification. For the YG mutant, an intermediate control plasmid was first created by whole plasmid PCR amplification to add the restriction site AflII to the N-terminus of the WT TMS of *SSS1*, creating the Leu Lys (LK) insertion. This plasmid was then modified to create an ApaI site, resulting in the Tyr Gly (YG) inversion at the C-terminus of the TMS. The F (phenylalanine) mutant was created by cassette mutagenesis: mutagenic oligonucleotides were annealed and subcloned as a KpnI-PsiI fragment into a vector containing the WT sequence of *SSS1* with a KpnI site inserted at the C-terminal end of the TMS. To create the CTSa mutant, the *SSS1* sequence excluding the C-terminal sequence (CTS) was subcloned in front of the CTS of ActA as a NheI-KpnI fragment. The TMSa mutant was constructed by placing the N-terminal *SSS1* sequence from the 5'UTR up to and including the N-terminal sequence, in front of the ActA TMS and CTS as an AflII-BglII fragment. The CTS of ActA was then replaced by the CTS of Sss1p as a KpnI-NheI fragment and the entire region was subcloned into a pRS313 derivative containing the 3'UTR of Sss1p. For the Tom5 mutant, primers containing NcoI and XhoI restriction sites were used to amplify the *TOM5* coding sequence, along with the 5'UTR, from purified yeast genomic DNA. The product was then subcloned as an NcoI-XhoI fragment into a psPUTK vector containing the Sss1p 3'UTR. From this construct, primers containing a 5' AflII and ApaI site, respectively, were used to amplify the *TOM5* TMS, CTS and the Sss1p 3'UTR and then subcloned into pRS313.

Myc-tagged plasmids containing WT and mutant Sss1p were created by inserting an oligonucleotide cassette (with flanking AgeI and NcoI sites) containing two copies of the myc tag (optimized for yeast codon usage) into the appropriate pRS313-derived Sss1p expression vectors. Plasmids for fluorescence microscopy were created by placing two coding regions in tandem into the yeast expression vector pRS425, a high copy vector containing a 2 micron origin of replication and a *LEU* selection marker (Christianson TW et. al., 1992). The coding regions

are comprised of GFP fused to the tail-anchor of yeast cytochrome b5 (Cyb5p) under the control of the *ADH* promoter (ADH-GFP-Cyb5TA), followed by the signal anchor of canine SRβ (the β subunit of the SRP receptor) fused to the N-terminus of mRFP under the control of the *GPD* promoter (GPD-SRβSA-mRFP).

Antibodies

To raise specific antibodies to Sss1p in rabbits, a pGEX derivative encoding full-length WT Sss1p fused to the carboxyl-terminus of Glutathione-S-Transferase was overexpressed in *E. coli* and purified by Glutathione-Sepharose 4B (GE Healthcare Bio-Sciences, Inc., Baie d'Urfe, Quebec, Canada) affinity chromatography. The resulting sera were tested and used at a 1:2000 dilution for immunoblots. Antisera raised against Sec63p (1:30,000), Sec62p (1:10,000), and prepro-alpha-factor (1:5000) were a gracious gift from Dr. Randy Schekman (University of California, Berkeley, CA). Antibodies against Sec72p were used as described [7]. Monoclonal antibodies raised against yeast ribosomal protein L3 (1:50,000) were a kind gift from Dr. Jonathon Warner (Albert Einstein College of Medicine, Bronx, NY). Anti-actin mouse monoclonal antibodies (1:30,000) were obtained from ICN Biomedical Ltd. (Montreal, Canada), and anti-yeast mitochondrial porin monoclonal antibodies (1:30,000) were from Molecular Probes Inc. (Eugene, OR). Hybridoma cell lines for the production of mouse ascites containing anti-myc monoclonal antibodies (used at 1:20,000) were obtained from ATCC. Primary antibodies were detected using HRP-conjugated secondary antibodies (Donkey anti-Rabbit, Donkey anti-Mouse, 1:30,000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA).

Yeast Strains, Growth Conditions and Cell Lysates

Yeast cells were grown in YPG (1% yeast extract, 1% peptone, 2% galactose) or YPD (as for YPG but 2% dextrose). Synthetic drop-out minimal medium (0.67% Yeast Nitrogen Base without amino acids, Sigma-Aldrich) was used for selection of auxotrophic strains, with appropriate amino acid additions (0.2%) and either galactose or dextrose (2%) as the carbon source. Media for plates were supplemented with 2% agar. Total cell lysates were prepared by centrifugation of 1 OD (600nm) unit of yeast cells, resuspension in 1X Loading Buffer (50mM Tris-Cl, pH 8.9, 2% SDS, 1mM EDTA, 10% v/v glycerol, 0.05 % bromphenol blue, and 250mM DTT), incubation at 65˚C for 10 min, and lysis by agitation on a Vortex mixer for 1 min after the addition of 0.12g of glass beads (425-600 μm, Sigma-Aldrich, Canada). Analysis by SDS-PAGE (10% Tricine) and immunoblotting confirmed the expression of each Sss1p mutant protein.