Extended Data Figures Legends

Extended Data Figure 1:

(a) RFP-Gas1 localization is not affected by mutants in SRP or NAC

Fluorescent micrographs of RFP-Gas1 confirm that it is not mislocalized when components of SRP, SRP receptor or NAC are compromised (control image can be found in Fig.1b). Scale bars throughout figure, 5 μm.

(b) SND mutants accumulate RFP-Gas1 in inclusions

Fluorescent micrographs of RFP-Gas1 confirm that its accumulation in *Δsnd* strains colocalize with the cytosolic inclusion marker, VHL-GFP.

(c) *SND* deletions do not have a non-specific effect on translation, targeting or translocation.

A fluorescently tagged SRP substrate (Hxt2-GFP) was mislocalized only in the temperature sensitive strain, *sec65-1*, when grown in the restrictive temperature of 37°C (under these conditions the cells are depleted for functional SRP). *SND* deleted strains display normal cell surface localization of Hxt2.

(d) Schemes of SND proteins

Schematic representation of the structural elements and topology-predictions of Snd1 (top), Snd2 (middle) and Snd3 (bottom). Numbers indicate the number of amino acids in the proteins.

(e) GFP-tagged SND proteins are functional

RFP-Gas1 is correctly localized in all GFP-tagged SND proteins, indicating that the tag does not disrupt their function and endogenous localization.

(f) An ortholog of Snd2 is present in canine microsomes

A mammalian ortholog of Snd2 (hSnd2) is present in canine pancreatic rough microsomes, which are routinely used as a source of mammalian ER proteins, as seen by immunoblotting with an antibody against hSnd2 which was shown to be specific in siRNA mediated gene silencing experiments.

(g) Endogenous hSnd2 is localized to the human rough ER

HEK293 cells were homogenized and subfractionated into various pellet (P) and supernatant (S) fractions. Fractions were analyzed by SDS-PAGE and immunoblotting. hSnd2 co-fractionated with the rough ER markers, Grp170 and Sec62, and the ribosomal protein uS3 but not with the nuclear and cytosolic proteins p68 and GAPDH. The areas of interest of luminescence images from a single western blot are shown.

For gel source data see Supplementary Figure 1.

Extended Data Figure 2:

(a) Snd2 and Snd3 form a complex together with the Sec61 translocon

BN-PAGE followed by 2nd dimension SDS-PAGE. Densitometry quantification revealed that Sec61 migrates in four distinct complexes, as well as a monomer. Interestingly, we found both Snd2 and Snd3 to reside together in two of these complexes, one of an approximate molecular mass of ~669 kDa, and a second supercomplex of a higher molecular mass. We postulate that the two Sec61/SND complexes may differ in size depending on the presence of additional auxiliary components. For gel source data see Supplementary Figure 1.

(b) Loss of each SND protein affects the localization of the others

Fluorescent micrographs showing that Snd2 is mislocalized upon deletion of *SND3* and Snd3 is mislocalized upon deletion of *SND1*, suggesting a functional dependence between the three proteins. Scale bars throughout figure, 5 μm.

(c) Growth rates reveal the genetic interactions between the *SND* genes

Heterozygous diploids of *Δsnd* were sporulated and tetrad-dissected to retrieve haploids. Tetrads obtained demonstrate an epistatic interaction between *SND1* and *SND2* mutants, and a synthetic sick interaction between *SND3* and the *SND1/2* mutants. As *SND3* is more than an order of magnitude more abundant than *SND1/2*, it is possible that this interaction is due to an independent cellular function.

(d) RFP-Gas1 localization is comparable between *SND* single and double mutants

Fluorescent micrographs of RFP-Gas1 in *SND* single and double mutants show that they are epistatic to each other in terms of their effect on targeting.

(e) Quantification of RFP-Gas1 mis-localization in *SND* double mutants

Quantification of the RFP-Gas1 mislocalization phenotype in *SND* single and double mutants (Extended Data Fig. 2d) reveals a buffering epistatic interaction between *SND* genes (100 cells were counted per strain).

Extended Data Figure 3: Substrate affinity to a targeting pathway depends on the position of its transmembrane domain

Quantification of the mislocalization phenotype in Fig. 2F and Fig. 2G confirms that re-positioning of a substrate's TMD can alter its dependence on the different targeting pathways.

Extended Data Figure 4:

(a) Overexpression of *SND* genes does not affect SRP levels

SND genes were over-expressed by growth on galactose in 30°C, and levels of Sec65 protein were measured by western-blot and normalized to Histone H3 loading control. No apparent change in *sec65-1* levels was detected, implying that the rescue observed in Fig. 3b-d is not due to increased SRP levels (data shown are means +/− s.e.m., n=3, biological replicates).

(b) Levels of SND proteins do not change in SRP-depleted cells

SND proteins were C-terminally tagged on the *sec65-1* background, and their levels were measured by western-blot when grown in either permissive or restrictive temperatures (30°C and 37°C respectively), and normalized to Actin loading control. No apparent change in Snd1 or Snd3 levels was observed. Snd2 levels were below detection threshold (data not shown).

(c) SND2 overexpression increases the translocation of DHCαF

Pulse radioactive metabolic labeling followed by DHCαF immunoprecipitation was used to measure the translocation rate of the DHCαF. *SND2* overexpression showed significantly higher translocation when compared to its repression by glucose,

regardless to the functional state of *sec65-1* (data shown are means +/− s.e.m. **p<0.01, ***p<0.001, by two-tailed Student's t-test, n=3, biological replicates).

For all gel source data see Supplementary Figure 1.

Extended Data Figure 5:

(a) Repression of *SND* genes is epistatic with *SEC72* and synthetic sick with *GET3*

Growth rate of strains with the *SND* genes expressed under the regulation of a repressible Tet-promoter were measured when grown on Tetracycline. The growth rate of *Δsec72 Tetp-SNDs* conditional double mutants is identical to the control, indicating that they are epistatic to one another. The *Δget3 Tetp-SNDs* conditional double mutants are sick, yet viable.

(b) Double deletion of *SND2* and *GET3* is lethal

Heterozygous diploids of *Δsnd2* and *Δget3* were sporulated and tetrad-dissected to retrieve haploids. Tetrads obtained demonstrate a synthetic lethal interaction between *SND2* and *GET3*.

(c) RFP-Gas1 translocation is moderately affected by *SND* single deletions

Pulse radioactive metabolic labeling followed by RFP-Gas1 immunoprecipitation was used to measure RFP-Gas1 translocation rates. Percentage of glycosylated ER and Golgi forms (indicated by 2 black lines) was reduced to 5% in *Δsec72,* while in *Δsnd1*, *Δsnd2* and *Δsnd3* it was reduced to 85%, 88% and 79% respectively (data shown are means (s.e.m.), n=3, biological replicates). All strains in this assay were attenuated for degradation with the *scl1-DAmP* proteasome hypomorphic allele.

(d) Verification of the glycosylated forms of RFP-Gas1

Pulse radioactive metabolic labeling followed by RFP-Gas1 immunoprecipitation was performed in the presence and absence of the glycosylation inhibitor Tunicamycin, allowing the identification of three forms of RFP-Gas1: Cytosolic, ER and Golgi (mature).

(e) CPY targeting is not affected by double mutants of the SND and GET pathways

Same methodology as in (c) was used to follow the SS-containing protein CPY in the conditional double mutant for *SND2/GET3*. A mild decrease in the glycosylated forms was observed in the *SND2* single mutant, however there was no translocation defect in the *GET3* single mutant or in the conditional double mutant. This result repeated in three independent biological repeats.

(f) MW of cytosolic CPY and translocated CPY (g-CPY)

CPY was metabolically labeled in a control strain and a partially translocated pool was visualized with a ladder to provide a size reference to (e).

(g) DHCαF translocation is not hampered by *SND* single deletions

Same methodology as in (c) was used to measure the translocation rate of the SRP-dependent substrate, DHCαF. In the temperature sensitive strain, *sec65-1,* in the restrictive temperature (37°C), there was no translocated substrate. *Δsnd1*'s translocation efficiency was comparable to the WT control. *Δsnd2* and *Δsnd3* translocation efficiency was significantly higher: ~160% glycosylated protein compared to the WT control (data shown are means (s.e.m.), n=3, biological repeats).

For all gel source data see Supplementary Figure 1.