ANALYSIS OF SSS1P: AN ESSENTIAL TAIL-ANCHOR PROTEIN IN YEAST

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AN ESSENTIAL TAIL-ANCHOR PROTEIN OF THE ER TRANSLOCON

IN THE YEAST *SACCHAROMYCES CEREVISIAE*

By

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Abstract

Sss1p is an essential component, along with Sec61p, of the protein conducting channel (PCC) in the ER of the yeast *Saccharomyces cerevisiae*. It belongs to a family of proteins termed tail-anchor (TA) proteins. The TA consists of a single hydrophobic sequence at the carboxyl-terminus which anchors the protein to the membrane in a Type II ($N_{cytoplasm}$-C_{lumen}) orientation. TA proteins are targeted to their membranes of function through an uncharacterized SRP-independent, post-translational mechanism. The targeting mechanism and function of Sss1p are not known. In this thesis, results will be presented from targeting and functional studies of Sss1p. Sss1p is predicted to contain an ER targeting signal similar to mammalian VAMP-1A. Disruption of this putative signal caused incomplete mislocalization of Sss1p to the mitochondria which did not affect yeast growth. Mutations in the TA of Sss1p had numerous effects. Yeast expressing these mutants showed diminished growth, a defect in co- and post-translational translocation, inefficient ribosome binding to Sec61p and the mislocalization of translocon components from light membranes (predominantly ER) to heavy membranes (predominantly mitochondria). It is argued that mutations in the TA of Sss1p disrupt the function of the protein, subsequently leading to the general defects listed above. Two possible functions for Sss1p are proposed: Sss1p is involved in forming the signal sequence binding pocket of the translocon and/or is essential for the integrity of the PCC.

Abbreviations

APX – peroxisomal ascorbate peroxidase
ATP – adenosine 5`-triphosphate
Bcl-2 – B cell lymphoma gene 2
bp – base pair
C – cushion
$CaCl_2$ – calcium chloride
Cb_5 – cytochrome b_5
CPIC – complete protease inhibitor cocktail
C-terminus – carboxyl terminus
CTS – carboxyl terminal sequence
cyt. – cytosol
DNA – deoxyribonucleic acid
dNTP – deoxynucleotide triphosphate
D.O. YEP – drop-out yeast extract peptone
DTT - dithiolthreitol
EDTA – ethylene diamine tetra acetic acid
ER – endoplasmic reticulum
g - grams
GTP – guanosine 5`-triphosphate
HCl – hydrochloric acid
HM – heavy membrane
kDa – kilo Dalton
KCl – potassium chloride
KOAc – potassium acetate
KOH – potassium hydroxide
L – litre
Ld – load
LM – light membrane
M – molar
mM – millimolar
$MgCl_2$ – magnesium chloride
$Mg(OAc)_2$ – magnesium acetate
mRNA – messenger RNA
M-site – ribosome membrane binding site
NAC – nascent chain associated complex
NaCl – sodium chloride
NaI – sodium iodide
NaOH – sodium hydroxide
nt - nucleotide
N-terminus – amino terminus
NTS – amino terminal sequence
$O.D._{600}$ – optical density at 600 nanometers

OGST – oligosaccharyl transferase
OMb – outer mitochondrial membrane cytochrome b_5
ORF – open reading frame
P – pellet
PCC – protein conducting channel
PCR – polymerase chain reaction
PEG – polyethylene glycol
PIN – protease inhibitor cocktail
pKar2p – pre-Kar2p
PKRM – puromycin/high salt-treated rough microsome
PMSF – phenyl methyl sulfonyl fluoride
ppαF – pre pro alpha factor
RM – rough microsome
RNA – ribonucleic acid
RNC – ribosome-nascent chain complex
SDS-PAGE – sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SPC – signal peptidase complex
SRP – signal recognition particle
SR – signal recognition particle receptor
SSS1 – suppressor of sec sixty one
TA – tail-anchor
Taq-Vent – mixture of Taq and Vent polymerase
TLB – tricene loading buffer
TMS – transmembrane sequence
Tom – translocase of the outer mitochondrial membrane
TRAM – translocating chain associated membrane protein
TX-100 – Triton X-100
μL – microlitre
UTR – untranslated region
μU – micro unit
VAMP – vesicle associated membrane protein
Wt – wild-type
xg – times gravity
YEP – yeast extract peptone
YEPD – yeast extract peptone dextrose
YEPG – yeast extract peptone galactose
YNB – yeast nitrogen base

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1. Introduction

In eukaryotic cells, many proteins must be integrated into or transported through the endoplasmic reticulum (ER) membrane to the lumen for modification and/or subsequent transport through the secretory pathway. The question of the mechanism for localizing such proteins to the ER, and the machinery required for transporting or integrating these targeted proteins has been vigorously studied both in yeast and mammalian systems for over 20 years.

Proteins destined for the ER carry a signal sequence at the amino terminus (N-terminus) of the protein. Signal sequence composition is very diverse, yet they characteristically are 30 amino acids in length, with the central 7 to 10 amino acids being hydrophobic. For some ER membrane proteins the first hydrophobic stretch or transmembrane sequence (TMS), made up of 20 or more amino acids, also acts as a signal sequence and is therefore termed a signal anchor. To date, there are two mechanisms by which signal sequence or signal anchor containing proteins are targeted to the ER. The first is the co-translational targeting pathway, which involves the signal recognition particle (SRP), while the second is the post-translational targeting pathway that operates independent of SRP.

While signal anchor proteins are targeted almost exclusively by SRP, a signal sequence protein can be targeted by either the co- or post-translational pathway. The relative hydrophobicity of the signal sequence determines the targeting pathway to be utilized, where proteins with a more hydrophobic signal sequence are favoured for co-translational targeting (Ng et al., 1996). Once targeted, substrates from both pathways utilize the protein translocation machinery, or 'translocon', to transport through or integrate into the ER membrane. The protein conducting channel (PCC) itself consists of three membrane proteins – in mammals they are Sec61α, Sec61β and Sec61γ. Yeast have two homologous PCCs – the primary PCC consists of Sec61p, Sbh1p and Sss1p while the secondary PCC is made up of the proteins Ssh1p, Sbh2p and Sss1p. The PCC, along with the membrane protein TRAM in mammals, and the membrane protein Sec63p along with the ER lumenal chaperone Kar2p in yeast, are necessary and sufficient for the co-translational transport or integration of signal sequence proteins at the ER. For post-translational transport, which has been characterized almost exclusively in yeast, the integral membrane proteins Sec62p and Sec71p, and the peripheral membrane protein Sec72p, are required along with the PCC, Sec63p and Kar2p.[1]

Recently, interest has arisen in a family of membrane proteins that appear to target to the ER, as well as to other cellular structures, independent of the pathways mentioned above. This family of 'tail-anchor proteins' (TA proteins), of which Sss1p - the focus of this study - belongs, are anchored to the membrane through a single TMS at the carboxyl-terminus (C-terminus) of the protein. Since the majority of the hydrophobic sequence of the protein would be located within the ribosome until translation is completed, these

[1] In this paper 'PCC' will be exclusively used to define the Sec61α, β and γ complex (Sec61p, Sbh1p, Sss1p and Ssh1p, Sbh2p, Sss1p complexes in yeast). 'Translocon' will refer to the PCC supplemented with other ER membrane or lumenal proteins essential for either co- or post-translational translocation through, or integration into, the ER membrane.

proteins are believed to be localized to their target membranes through an SRP-independent, post-translational targeting mechanism (Anderson et al., 1983). The purpose of this study was two-fold: to study Sss1p to (1) gain insight into its targeting pathway and (2) understand its function as an integral component of the PCC.

This introduction will present an analysis of both the co- and post-translational ER targeting pathways, the structure and function of the PCC, and will summarize what is currently known of the targeting pathway for TA proteins (For other reviews, see Millman & Andrews, 1997; Stirling, 1999; Wattenberg & Lithgow, 2001)

1.1. Co-translational targeting

1.1.1. SRP targeting of signal sequence proteins to the ER membrane

The mammalian SRP is a cytoplasmic ribonucleoprotein consisting of six polypeptides and a small ribonucleic acid (RNA) molecule. The 300 nucleotide (nt) 7SL RNA (519 nt scR1 RNA in yeast; Hann & Walter, 1991) acts as a scaffold; SRP19 (Sec65p in yeast; Stirling & Hewitt, 1992) physically binds to the center of the RNA scaffold which allows SRP54 (Srp54p in yeast; Hann et al., 1989) to bind (Siegel & Walter, 1988a; Hann et al., 1992). Two heterodimers, SRP72/SRP68 and SRP14/SRP9 (Srp72p/Srp68p and Srp14p/uncharacterized 7 kDa protein in yeast; Brown et al., 1994) respectively bind near the 3` and 5` ends of the RNA scaffold to complete the assembly of SRP (Siegel & Walter, 1988a). Yeast SRP also contains a unique protein, not found in mammalian SRP, named Srp21p (Brown et al., 1994). Genetic studies in yeast have determined that Srp72p, Srp68p, Srp21p and Srp14p are essential for the stability of SRP (Brown et al., 1994).

A model for SRP-dependent targeting to the ER membrane is found in Figure 1. As a cytoplasmic ribosome begins translation of a messenger RNA (mRNA), the elongating nascent polypeptide, or 'nascent chain', contacts the heterodimeric nascent-polypeptide-associated complex (NAC), which is associated with ribosomes. NAC, consisting of an α and β subunit (Edg2p and Edg1p in yeast; George et al., 1998), can be cross-linked to nascent polypeptides that are 17 to 100 amino acids in length (Wiedmann et al., 1994; Wang et al., 1995), however the efficiency of these cross-links are significantly diminished as the nascent chain length extends past 30 amino acids (Wang et al., 1995). It is believed that NAC is a regulator of co-translational targeting, in that it both blocks SRP binding to nascent chains lacking a signal sequence and covers the membrane binding site (M-site) on the ribosome, thus preventing the mistargeting of non-signal sequence proteins to the ER (Wiedmann et al., 1994; Wang et al., 1995; Lauring et al., 1995a; Lauring et al, 1995b; Moller et al., 1998a, Moller et al., 1998b). This role for NAC has been experimentally challenged (Neuhof et al., 1998; Raden & Gilmore, 1998), and given that the NAC can protect nascent chains up to 30 amino acids in length from proteolysis (Wang et al., 1995), some feel that the NAC may simply form the ribosome exit tunnel. Such a structure would allow the efficient folding of N-terminal targeting signals (George et al., 1998), including the signal sequence.

Figure 1

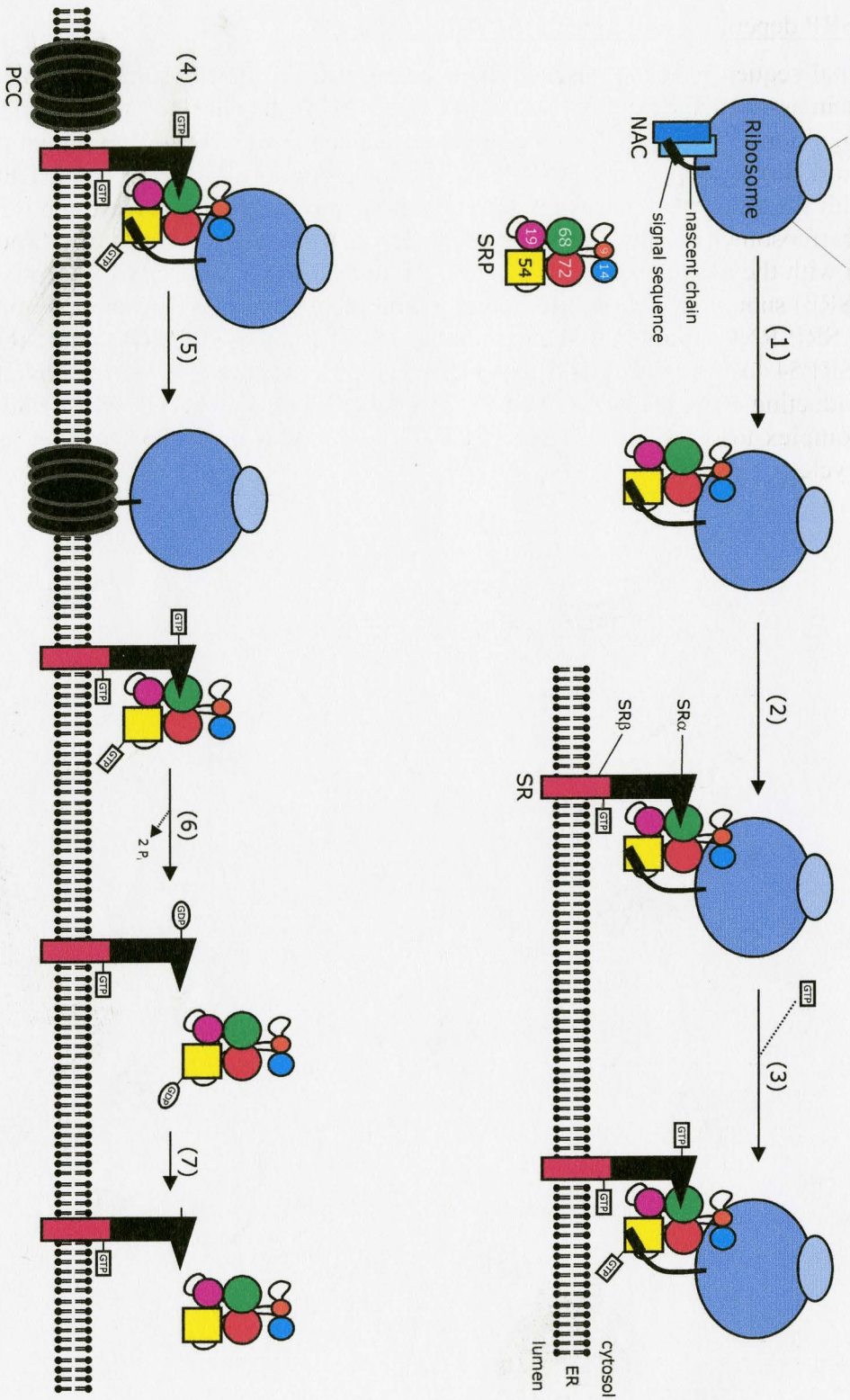

30S subunit
60S subunit
Ribosome
NAC
nascent chain
signal sequence
SRP
19
54
68
72
14
(1)
(2)
(3)
(4)
(5)
(6)
(7)
SR
SRα
SRβ
GTP
GDP
2 Pi
PCC
cytosol
ER
lumen

Figure 1: SRP dependent targeting of the RNC to the ER

As the signal sequence of the nascent chain emerges from the ribosome, it contacts the nascent chain associated complex (NAC). (1) The NAC is displaced by the binding of the signal recognition particle (SRP) to the signal sequence through an interaction with the 54 kilo Dalton (kDa) subunit of SRP (SRP54). The interaction of the 9 and 14 kDa subunits of SRP with the ribosome causes an arrest in elongation of the nascent chain. (2) SRP targets the ribosome-nascent chain complex (RNC) to the ER membrane through an interaction with the SRP receptor (SR). The SRP receptor is composed of an alpha (SRα) and beta (SRβ) subunit. SRα interacts directly with the 68 and 72 kDa subunits of SRP to anchor the SRP-RNC complex to the membrane. (3) The binding of SRP to the SR causes SRα and SRP54 to bind GTP. (4-5) SRβ then mediates the transfer of the RNC to the protein conducting channel (PCC). (6) SRα and SRP54 hydrolyze GTP, which allows the SRP-SR complex to dissociate. (7) Nucleotide is removed from SRP54 to reset the SRP targeting cycle.

Work in yeast has shown that SRP samples a nascent chain for a signal sequence as it emerges from the ribosome (Ogg & Walter, 1995). If a signal sequence is present, SRP binds to it, displacing NAC from the nascent chain (Wiedmann et al., 1994; Lauring et al., 1995a, Moller et al., 1998a, Moller et al., 1998b). There is evidence that at this stage SRP now covers the ribosome M-site, preventing direct binding of the ribosome to the ER membrane (Moller et al., 1998b; Pool et al., 2002). Binding of SRP to a signal sequence is mediated by SRP54 (Kurzchalia et al., 1986; Krieg et al., 1986; Wiedmann et al., 1987; Siegel & Walter, 1988a; Zopf et al., 1990; Hauser et al., 1995), through an interaction with the C-terminal methionine-rich domain (Zopf et al., 1990; High & Dobberstein, 1991.) In addition, SRP54 is the only GTP binding protein in the SRP, and must be in its empty state (lacking a nucleotide) to bind a signal sequence (Miller et al., 1993; Rapiejko & Gilmore, 1997).

Once bound to a signal sequence, SRP mediates a pause in the elongation of the nascent polypeptide (Walter & Blobel, 1981; Wolin & Walter, 1989) through what is thought to be a direct interaction of the SRP14/SRP9 heterodimer with the ribosome (Siegel & Walter, 1988a). This elongation arrest is believed to be important for SRP-mediated targeting of the ribosome-nascent chain complex (RNC) to the ER *in vivo*, since it has been shown that ribosomes with long nascent chains are inefficiently targeted (Siegel & Walter, 1988b).

Targeting of the SRP-RNC to the ER occurs through an interaction with the SRP receptor (SR) on the membrane. The SR is a heterodimer of two GTP-binding proteins (Connolly & Gilmore, 1989; Miller et al., 1995, Young et al., 1995). The β-subunit (SRβ - Srp102p in yeast) is a membrane protein that must be bound to nucleotide (GTP or GDP) in order to physically anchor the soluble α-subunit (SRα - Srp101p in yeast) to the ER membrane (Miller et al., 1995; Ogg et al., 1998; Legate et al., 2000). Evidence suggests that the SRP72/SRP68 heterodimer facilitates SRP binding to the SR (Siegel & Walter, 1988; Rapiejko & Gilmore, 1992; Miller et al., 1995; Rapiejko & Gilmore, 1997) through a nucleotide-independent interaction with SRα (Rapiejko & Gilmore, 1992; Rapiejko & Gilmore, 1997). SRP binding to the SR increases the affinity of SRP54 and SRα for GTP, which stabilizes the SRP/SR complex (Rapiejko & Gilmore, 1992; Miller et al., 1993; Rapiejko & Gilmore, 1997) and is also believed to remove SRP from the M-site on the ribosome (Moller et al., 1998b; Pool et al., 2002).

Concomitantly, GTP-bound SRβ co-ordinates signal sequence release from SRP, and the subsequent transfer of the RNC to the translocon, through a poorly understood mechanism involving the interaction of SRβ with the ribosome and the translocon (Connolly & Gilmore, 1989; Connolly et al., 1991; Rapiejko & Gilmore, 1992; Bacher et al., 1999; Song et al., 2000; Fulga et al., 2001). Surprisingly, yeast Srp102p (SRβ) does not need its TMS in order to perform its function, as nearly 50% of the truncated protein remains bound to the ER membrane (Ogg et al., 1998). GTP hydrolysis by SRP54 and SRα lead to the dissociation of SRP from the SR (Connolly et al., 1991; Rapiejko & Gilmore, 1997), effectively re-setting the SRP-SR targeting pathway.

1.1.2. Translocation/Integration of signal sequence proteins via the ER resident translocon

The mammalian translocon is a dynamic complex. It is composed of the membrane proteins Sec61α (Gorlich et al., 1992b), Sec61β, and Sec61γ (Hartmann et al., 1994) which form the PCC. Other components include the 'translocating chain associated membrane protein' (TRAM) (Gorlich et al., 1992a), oligosaccharyl transferase (OGST), the signal peptidase complex (SPC) and potentially the ER lumenal heat shock protein BiP (Gorlich & Rapoport, 1993; Nicchitta & Blobel, 1993). In yeast the primary PCC is composed of the Sec61α, β and γ homologs named Sec61p (Deshaies & Schekman, 1987), Sbh1p (Panzner et al., 1995) and Sss1p (Esnault et al., 1993). Included is the membrane protein Sec63p (Rothblatt et al., 1989), the BiP homolog Kar2p (Vogel et al., 1990), the lumenal heat shock protein Lhs1p (Craven et al., 1996) and two Lhs1p/Kar2p binding proteins named Sil1p and Sls1p (Kabani et al., 2000; Tyson & Stirling, 2000).

There is overwhelming experimental evidence to support the role of membrane proteins in forming an aqueous channel used for the co-translational translocation or integration of signal sequence polypeptides at the ER. Early work in yeast provided genetic evidence for the involvement of membrane proteins in the transport across and integration of polypeptides into the ER membrane (Deshaies & Schekman, 1987; Rothblatt et al., 1989; Stirling et al., 1992). These membrane proteins were shown to exist in a complex (Deshaies et al., 1991; Esnault et al., 1994; Panzner et al., 1995) that could be re-constituted into liposomes from purified components *in vitro* (Gorlich & Rapoport, 1993). Electrophysiological experiments demonstrated that the removal of the nascent chain from membrane bound ribosomes by inducing premature chain termination with puromycin opened a channel which was permeable to ions (Simon & Blobel, 1991). Proteolysis experiments have shown that 30 to 40 amino acids of a nascent chain, which would be long enough to span the membrane, are protected by membrane bound protein components (Matlack & Walter, 1995). Fluorescently labeled nascent chains have been shown to move through a non-cytoplasmic aqueous environment during translocation through the ER (Crowley et al., 1993; Crowley et al., 1994). Sec61α, β, γ and TRAM can be chemically cross-linked to secretory (High et al., 1993b; Mothes et al., 1994; Nicchitta et al., 1995) and membrane proteins (High et al., 1993a; Oliver et al., 1995; Do et al., 1996; Laird & High, 1997) during their translocation. Sec61α, β and γ are also tightly associated with ribosomes (Gorlich et al., 1992a; Gorlich & Rapoport, 1993; Panzner et al., 1995) and are protected from proteolytic degradation due to this interaction (Kalies et al., 1994; Murphy III et al., 1997; Potter & Nicchitta, 2000). Finally, electron microscopy has demonstrated that the purified complex of Sec61α, β and γ form oligomeric rings with a central pore when bound to ribosomes (Hanein et al., 1996; Beckmann et al., 2001). All of this data is consistent with the transit of polypeptides across or into the membrane of the ER being mediated by a translocon. A model for co-translational transport/integration of proteins via a translocon in the ER is presented in Figure 2.

A free translocon, with an aqueous pore diameter of 9-15 angstroms, is sealed at the lumenal side of the ER by BiP (no studies have been undertaken to determine if this function for Kar2p is conserved in yeast) (Hamman et al., 1998), which appears to be recruited to the translocon through an Sls1p-stimulated (Kabani, et al., 2000) physical

Figure 2

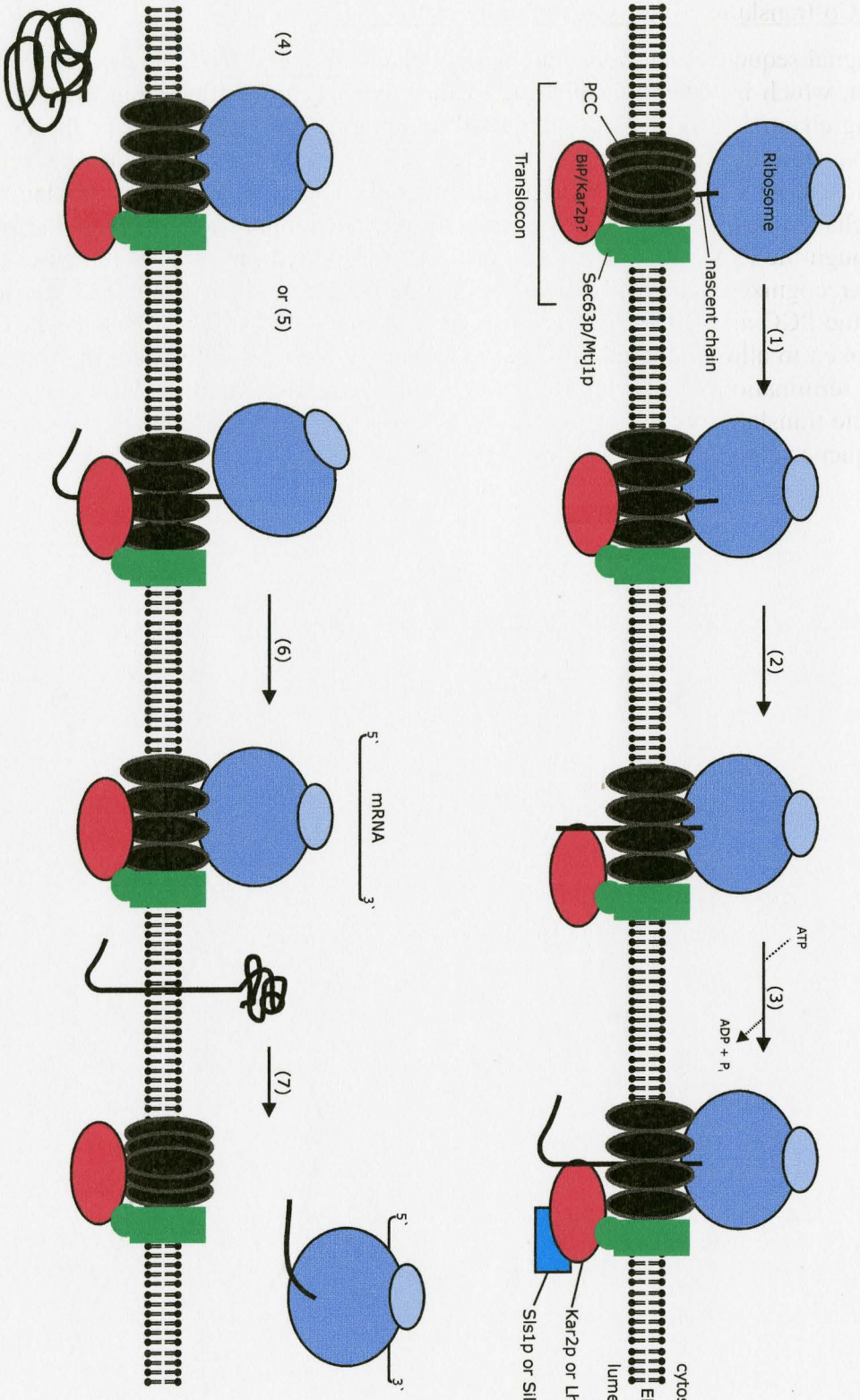

Ribosome
nascent chain
PCC
BiP/Kar2p?
Sec63p/Mtj1p
Translocon
(1)
(2)
ATP
(3)
ADP + P$_i$
cytosol
ER
lumen
Kar2p or Lhs1p
Sls1p or Sil1p
(4)
or (5)
(6)
(7)
5`
mRNA
3`
5`
3`

Figure 2: Co-translational transport of proteins to the ER

(1) The signal sequence of a ribosome-nascent chain complex (RNC) is recognized by the translocon, which initiates tight binding of the ribosome to, and opening of, the protein conducting channel (PCC). At this time BiP maintains the lumenal seal of the PCC. (2) Translation continues until the nascent chain elongates to an approximate size of 70 amino acids, at which point the lumenal seal is broken. (3) Acting as a molecular ratchet, lumenal chaperones utilize ATP to ensure the unidirectional movement of the nascent chain through the PCC. (4) The nascent chain is transported into the ER lumen or (5) the ribosome recognizes a transmembrane sequence on the nascent chain and transmits a signal to the PCC and BiP, which reforms the lumenal seal. (6) The ribosome-PCC tight seal is broken to allow integration of the membrane protein laterally from the translocon. (7) Upon termination of translation, the ribosome remains bound to the translocon and may initiate translation of a new messenger RNA (mRNA). If the mRNA encodes a non-signal sequence containing protein, the ribosome detaches from the translocon.

interaction with the lumenal J-Domain of Sec63p in yeast (Sadler et al., 1989; Corsi & Schekman, 1997; McClellan et al., 1998; Misselwitz et al., 1999) or Mtj1p in mammals (Dudek et al., 2002). This seal is essential for maintaining the permeability barrier of the ER membrane (Hamman et al., 1998). The RNC is transferred from the SRP-SR complex to the free translocon where the signal sequence is recognized by the PCC and inserted into the aqueous pore, initiating a tight junction between the ribosome and the PCC (Crowley et al., 1993; Jungnickel & Rapoport, 1995; Raden et al., 2000). Ribosome binding causes a conformational change in the translocon, opening the aqueous pore to a size of 40-60 angstroms (Hamman et al., 1997) without compromising the permeability barrier (Hamman et at., 1998).

With the removal of SRP from the RNC, elongation of the nascent chain continues and the signal sequence is cleaved from the nascent polypeptide by the SPC. Driven by elongation, a soluble polypeptide traverses the aqueous pore. When the nascent chain length reaches 70 amino acids the lumenal seal is broken and the peptide may pass into the lumen of the ER (Crowley et al., 1994); the permeability barrier is maintained by the tight ribosome-PCC seal. The unidirectional movement of the nascent chain through the pore is ensured by BiP/Kar2p (Brodsky et al., 1995; Lyman and Schekman, 1995; Young et al., 2001) as well as Lhs1p in yeast (Tyson & Stirling, 2000). In an apparent ATP-dependent, Sec63p/Sil1p/Sls1p-stimulated mechanism, BiP/Kar2p and Lhs1p bind to translocating nascent chains (Hamilton et al., 1999; Kabani et al., 2000; Tyson & Stirling, 2000), acting like a molecular ratchet (Matlack et al., 1999). The many functions of BiP/Kar2p in translocation appear to be specific, as it cannot be replaced by cytoplasmic or mitochondrial heat shock proteins (Brodsky et al., 1993; Brodsky et al., 1998).

The translocon utilizes a different mechanism for the co-translational integration of membrane proteins. The TMS of membrane proteins are first recognized inside the ribosome, which transmits a signal to the translocon, preparing it for integration (Liao et al., 1997). Once the TMS enters the pore, the translocon opens laterally to allow diffusion of the TMS into the ER lipid bilayer (Martoglio et al., 1995; Heinrich et al., 2000). It has been shown that the ribosome-translocon seal is broken during membrane protein integration (Liao et al., 1997), with BiP maintaining the permeability barrier by sealing the lumenal end of the translocon (Haigh & Johnson, 2002).

Recent evidence suggests that upon completion of protein synthesis the ribosome remains bound to the translocon and can initiate the synthesis and transport of a signal sequence containing protein while still on the membrane. These membrane bound ribosomes can also initiate the synthesis of non-signal sequence proteins, however doing so leads to ribosome detachment from the ER (Potter & Nicchitta, 2000; Potter & Nicchitta, 2002). With ribosome detachment, the translocon pore closes (Simon & Blobel, 1991) and is now ready for another round of SRP-dependent co-translational targeting.

1.2. Post-translational targeting

The discovery that yeast can target signal sequence-proteins to the ER post-translationally (Waters and Blobel, 1986) and that neither yeast SRP nor the SR are essential for viability (Hann & Walter, 1991; Ogg et al., 1992) led to the theory that yeast must also have evolved an SRP-independent targeting mechanism. Yeast genetic and *in*

vitro experiments confirmed the role of the cytoplasmic heat shock proteins Ssa1p and Ydj1p in a post-translational targeting mechanism (Chirico et al., 1988; Caplan et al., 1992; Becker et al., 1996; McClellan & Brodsky, 2000; Plath and Rapoport, 2000). Similar experiments also confirmed the involvement of components of the co-translational translocon, namely Sec61p, Sec63p, Sss1p and Kar2p (Deshaies & Schekman, 1987; Rothblatt et al., 1989; Esnault et al., 1993; Brodsky et al., 1995; Lyman & Schekman, 1995; McClellan et al., 1998).

Three other ER associated proteins were also identified as playing a role in post-translational transport. They include the membrane proteins Sec62p (Rothblatt et al., 1989) and Sec71p, as well as the peripheral membrane protein Sec72p (Green et al., 1992). Yeast expressing mutants of these proteins also show defects in the post-translational translocation or integration of protein substrates at the ER (Deshaies & Schekman, 1989; Green et al., 1992; Kurihara & Silver, 1993; Feldheim et al., 1993; Fang & Green, 1994; Feldheim & Schekman, 1994).

There is a great deal of evidence demonstrating that Kar2p, Sec62p, Sec63p, Sec71p and Sec72p interact with the PCC and function as a post-translational translocon. Sec62p has been shown to interact with Sec61p and Sec63p both *in vitro* and *in vivo* (Deshaies & Schekman, 1990; Wittke et al., 2000; Willer et al., 2003). Sec71p is a high-copy suppressor of a thermo-sensitive *sec63* allele (Kurihara & Silver, 1993), and *sec71* mutations are synthetically lethal in combination with *sec61*, *sec62*, *sec63* and *kar2* mutations (Kurihara & Silver, 1993; Fang & Green, 1994), suggestive of a physical interaction. An unstable *sec71* mutant leads to the degradation of Sec72p, indicative of a physical interaction between the two proteins (Fang & Green, 1994). Both Sec71p and Sec72p can be chemically cross-linked to Sec61p and Sec63p (Feldheim et al., 1992; Fang & Green, 1994). Chemical cross-linking and immunoprecipitation studies demonstrated that Sec61p, Sec62p, Sec63p, Sec71p and Sec72p exist as a dynamic complex (Deshaies et al., 1991). A Sec61p/Sec62p-independent sub-complex containing Sec63p, Sec71p, Sec72p and Kar2p was also identified (Brodsky & Schekman, 1993); interestingly, all of the members of this sub-complex have been implicated in yeast nuclear import (Sadler et al., 1989) and nuclear fusion (karyogamy) during mating (Ng & Walter, 1996; Brizzio et al., 1999).

There are three sets of experiments that provide the most convincing data to support the function of the above mentioned proteins as a post-translational translocon. (1) Experiments have shown post-translational signal sequence-protein substrates can be chemically cross-linked to Sec61p, Sec62p, Sec63p and Sec71p as they are transported across the ER membrane (Musch et al., 1992; Sanders et al., 1992; Pilon et al., 1998; Plath et al., 1998). (2) The minimal components required for post-translational transport across the membrane of reconstituted proteoliposomes *in vitro* includes the PCC, Sec62p, Sec63p, Sec71p, Sec72p and Kar2p (Brodsky & Schekman, 1993; Panzner et al., 1995). (3) *In vitro* and *in vivo* work has shown that Sec62p, Sec71p and Sec72p, along with Sec61p (Plath et al., 1998), form a post-translational signal sequence receptor on the ER membrane that appears to require Sec63p, Kar2p and ATP for the effective transfer of the signal sequence into the PCC (Feldheim & Schekman, 1994; Lyman & Schekman, 1997;

Pilon et al., 1998; Dunnwald et al., 1999). The model for post-translational targeting to the ER that emerges from the evidence listed here is outlined in Figure 3.

According to the model, a signal sequence containing peptide is synthesized and released from a cytoplasmic ribosome, where it is bound in an ATP-dependent manner by the cytoplasmic heat shock protein complex of Ssa1p and Ydj1p. These interactions keep the polypeptide in a translocation competent state. The signal sequence is apparently exposed, and targets the polypeptide-heat shock protein complex to the ER through an interaction with the signal sequence receptor, composed of Sec61p, Sec62p, Sec71p and Sec72p. An interaction of this complex with Sec63p and Kar2p triggers an ATP-dependent transfer of the polypeptide into the PCC, where transport through or integration into the ER membrane is carried out, presumably by a similar mechanism as co-translational substrates. How the polypeptide moves through the pore to contact Kar2p and the maintenance of the permeability barrier during post-translational transport have not been investigated. Although this targeting pathway has been exclusively studied in yeast, recently mammalian homologs of Sec62p and Sec63p have been discovered (Meyer et al., 2000; Tyedmers et al., 2000), hinting that a post-translational targeting pathway may exist in multicellular eukaryotes.

1.3. The protein conducting channel

Both co- and post-translational targeting pathways utilize a PCC. In addition to translocation of polypeptides from the cytoplasm into the lumen of the ER, the PCC has been implicated in the export of misfolded proteins from the ER lumen to the cytoplasm for degradation (Wiertz et al., 1996; Pilon et al., 1997). As previously mentioned, the PCC is composed of Sec61α, Sec61β and Sec61γ in mammals. In yeast there are two PCCs. The primary PCC is composed of Sec61p, Sbh1p and Sss1p (for simplicity the yeast nomenclature will now be exclusively used – Figure 4).

Sbh1p is a non-essential 10 kDa TA protein (Panzner et al., 1995, Toikkanen et al., 1996) that has been shown to bind ribosomes *in vitro* (Levy et al., 2001) and contact nascent polypeptide chains early in translocation, before they reach Sec61p (Laird & High, 1997). Sbh1p has also been shown to interact with the SPC (Kalies et al., 1998; Antonin et al., 2000), and is theorized to recruit the SPC to the translocon. In the yeast strain *Yarrowia lipolytica*, Sbh1p has been shown to interact with the ER lumenal chaperone calnexin and therefore may be important in linking nascent chain folding with translocation (Boisrame et al., 2002).

Sss1p is a 9 kDa TA protein essential for yeast viability (Esnault et al., 1993); it was identified as a high-copy suppressor of a thermo-sensitive *sec61* mutant that physically stabilizes the mutant protein (Esnault et al., 1994). Depletion of Sss1p leads to a severe defect in co- and post-translational translocation (Esnault et al., 1993), however its specific role in polypeptide translocation has not been elucidated. Numerous functions have been suggested for Sss1p – they include a potential role in maintaining the permeability barrier by acting as a translocon 'plug', acting as a place-holder for signal peptides (Plath et al., 1998) and functioning as a structural protein facilitating the oligomerization of the PCC (Wilkinson et al., 1997).

Figure 3

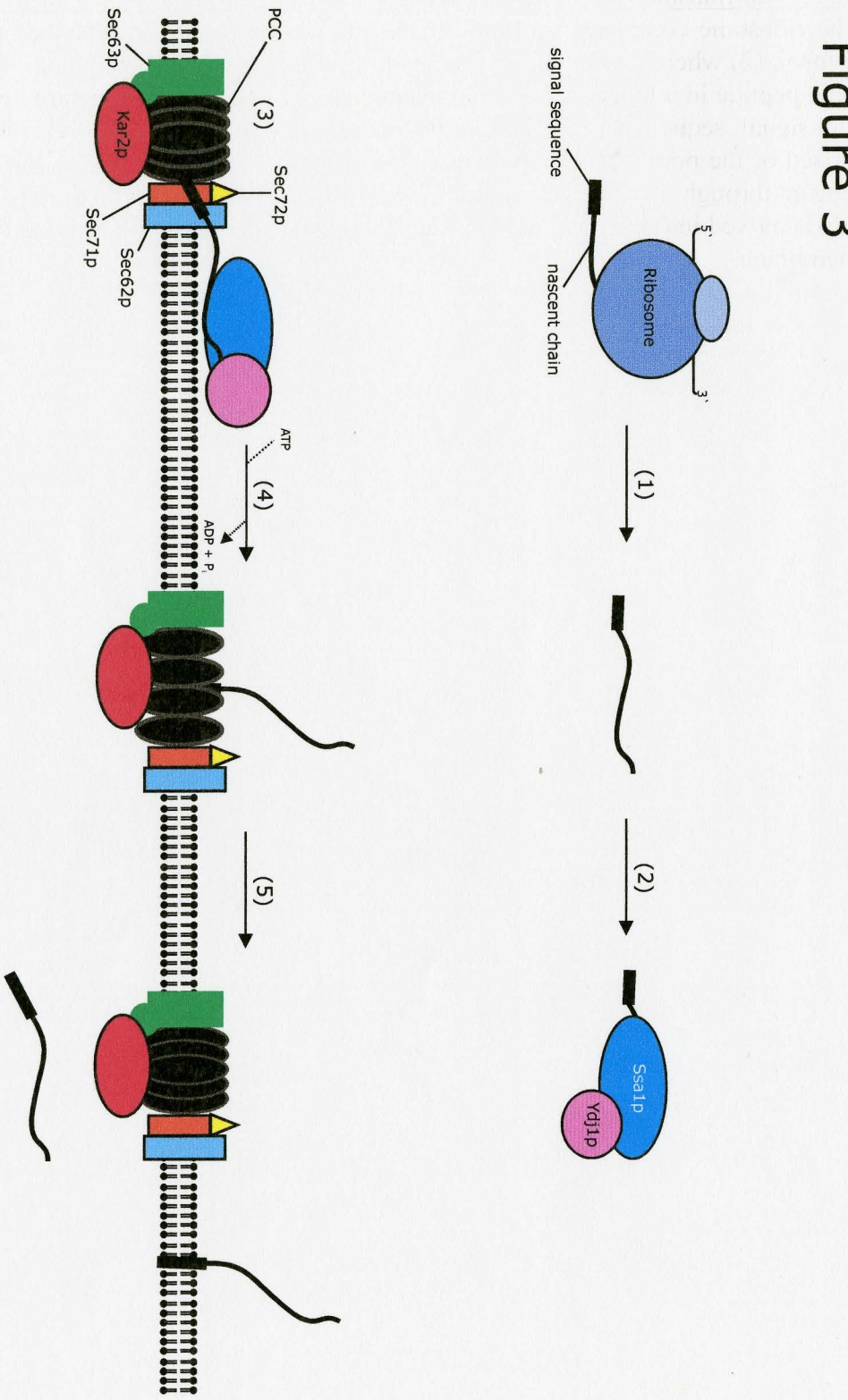

signal sequence
nascent chain
Ribosome
5'
3'
(1)
(2)
Ssa1p
Ydj1p
PCC
Sec63p
Kar2p
(3)
Sec72p
Sec71p
Sec62p
ATP
(4)
ADP + P
(5)

Figure 3: Post-translational transport of signal sequence-proteins to the ER in yeast
(1) The ribosome completes translation and releases the newly synthesized protein into the cytosol, (2) where it is bound by the cytoplasmic chaperones Ssa1p and Ydj1p, which keep the peptide in a translocation-competent state. (3) The complex is targeted to the ER by the signal sequence (SS), where it binds the signal sequence receptor complex comprised of the proteins Sec61p, Sec62p, Sec71p and Sec72p. Kar2p is recruited to the translocon through its interaction with Sec63p. (4) With the hydrolysis of ATP, the peptide is moved into the pore and (5) is either translocated through, or integrated into the ER membrane.

Figure 4 Predicted Topology of the Components of the Protein Conducting Channel (PCC)

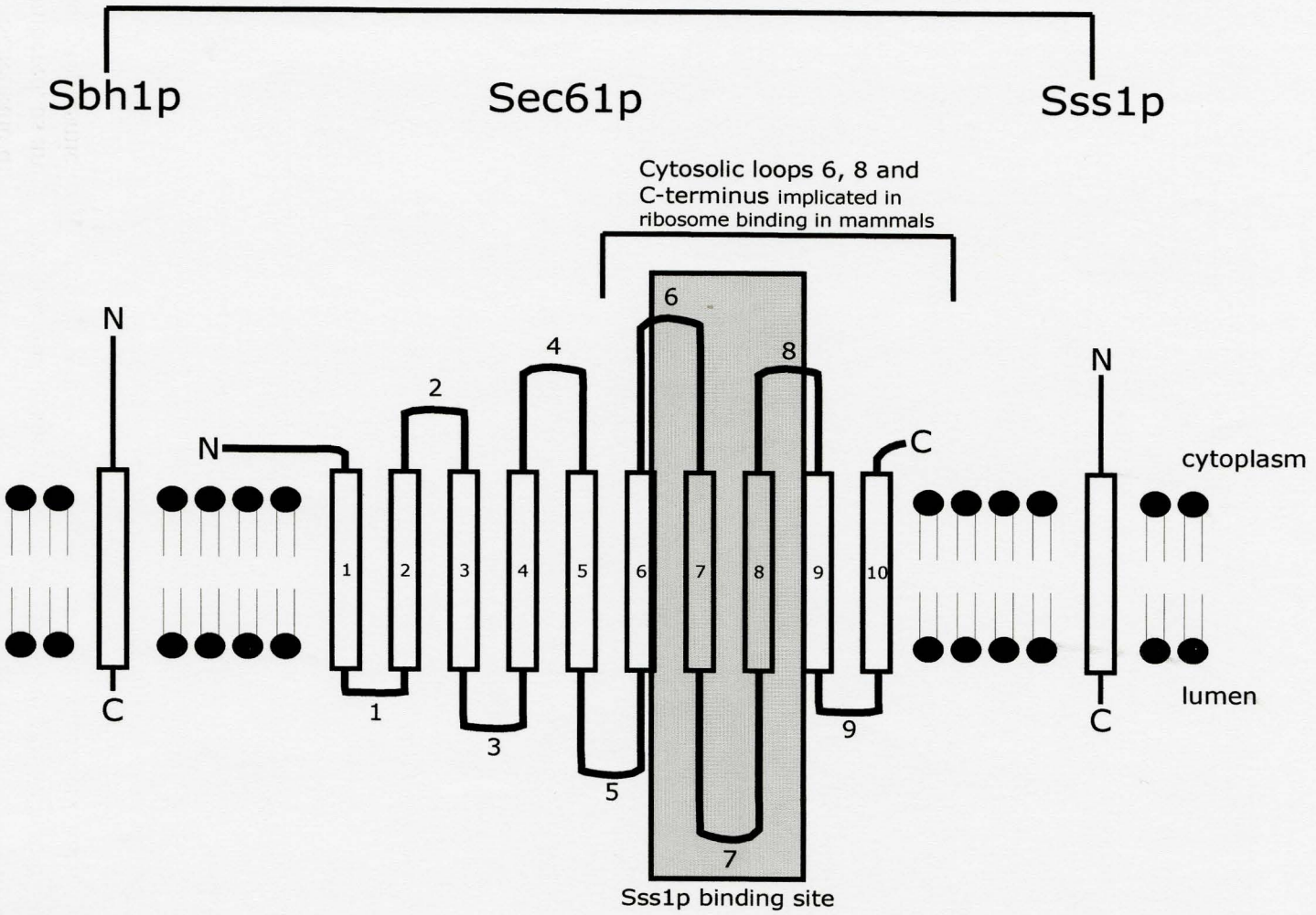


Figure 4: The predicted membrane topology of the components of the Protein Conducting Channel (PCC)

Schematic diagram of the PCC. The transmembrane sequences (TMS) of Sec61p are numbered, as are the lumenal and cytoplasmic loops. The Sss1p binding site is boxed, and the cytoplasmic loops of Sec61p involved in ribosome binding are indicated.

Sec61p is an essential 54 kDa protein that spans the membrane 10 times (Wilkinson et al., 1996). It is likely the major constituent of the aqueous channel. The functional domains of Sec61p have been systematically analyzed. TMS2, TMS3 and TMS7 have been implicated in post-translational translocation (Plath et al., 1998; Wilkinson et al., 2000). TMS3, TMS4 and cytoplasmic loop 6 are important for translocation into the ER lumen (Pilon et al., 1998; Raden et al., 2000; Wilkinson et al., 2000), while TMS3, TMS4 and lumenal loop 7 have a demonstrated role in the export of misfolded proteins from the ER lumen to the cytosol (Pilon et al., 1997). Chemical cross-linking experiments have shown that amino acid residues 232 to 406, which includes TMS6-8, cytoplasmic loops 6 and 8, and lumenal loop 7, form the binding site for Sss1p (Wilkinson et al., 1997).

Mutant Sec61p proteins not complexed with Sss1p are proteolytically degraded by the ubiquitin-proteasome pathway (Biederer et al., 1996); the degradation of mutant Sec61p subsequently destabilizes Sss1p (Esnault et al., 1994; Biederer et al, 1996). Proteolysis experiments have shown that TMS6, TMS8 and the C-terminus of mammalian Sec61p are involved in ribosome binding (Raden et al., 2000). This study found that ribosome binding requires oligomeric Sec61p, a conclusion that appears to have been confirmed by cryo-EM reconstruction of RNC-PCC complexes in yeast. The latter work showed asymmetric binding of the ribosome to a trimer of Sec61p/Sbh1p/Sss1p heterotrimers through ribosomal RNA (rRNA) and protein components of the large ribosomal subunit (Prinz et al., 2000a; Beckmann et al., 2001).

The secondary PCC in yeast consists of Ssh1p (Sec61p homolog), Sbh2p (Sbh1p homolog) and Sss1p (Finke et al., 1996). Ssh1p shares 30% total amino acid identity with Sec61p, and 40% identity in the cytoplasmic loops (Finke et al., 1996). Sbh2p shares 53% identity with Sbh1p (Toikkanen et al., 1996). Neither Ssh1p nor Sbh2p are essential (Finke et al., 1996; Toikkanen et al., 1996), and there are conflicting reports surrounding the *in vivo* effects of Ssh1p depletion (Finke et al., 1996; Wilkinson et al., 2001; Jeff Brodsky, personal communication). The Ssh1p complex has been found associated with ribosomes although it is not a major ribosome binding site, showing four-fold less ribosome binding than the main ribosome receptor Sec61p (Prinz et al., 2000b). In addition, the Ssh1p complex appears to be exclusively involved in co-translational targeting, as it preferentially transports co-translational substrates (Wittke et al., 2002) and is not found associated with any proteins involved in post-translational targeting (Finke et al., 1996).

1.4. Tail-anchor proteins

1.4.1. Targeting mechanisms of tail-anchor proteins

As mentioned previously, Sss1p is the focus of the present study. Sss1p belongs to a family of proteins termed tail-anchor (TA) proteins. The TA consists of a single hydrophobic sequence at the C-terminus which anchors the protein to the membrane in a Type II ($N_{cytosol}$-C_{lumen}) orientation. TA proteins have been found in most cellular membranes, including the ER, mitochondria, peroxisomes, nuclear envelope, Golgi apparatus, vacuole, endosome and plasma membrane. It has been suggested that most TA proteins are either targeted exclusively to the mitochondria, or are first targeted to the ER

and then sorted to their final destination (Linstedt et al., 1995; Rayner & Pelham, 1997; Mullen et al., 1999). Certain studies have found that sorting of ER targeted TA proteins appears to be dependent on the length of the C-terminal TMS (Whitley et al., 1996; Rayner & Pelham, 1997; Honsho et al., 1998). However a recent study in yeast has discovered that ER destined TA proteins are first targeted to the membrane by the relatively hydrophobic TMS and then sorted to their final destination by a signal in the cytoplasmic N-terminus (Beilharz et al., 2003). This same group also found that mitochondrial TA proteins require a less hydrophobic TMS to be properly targeted.

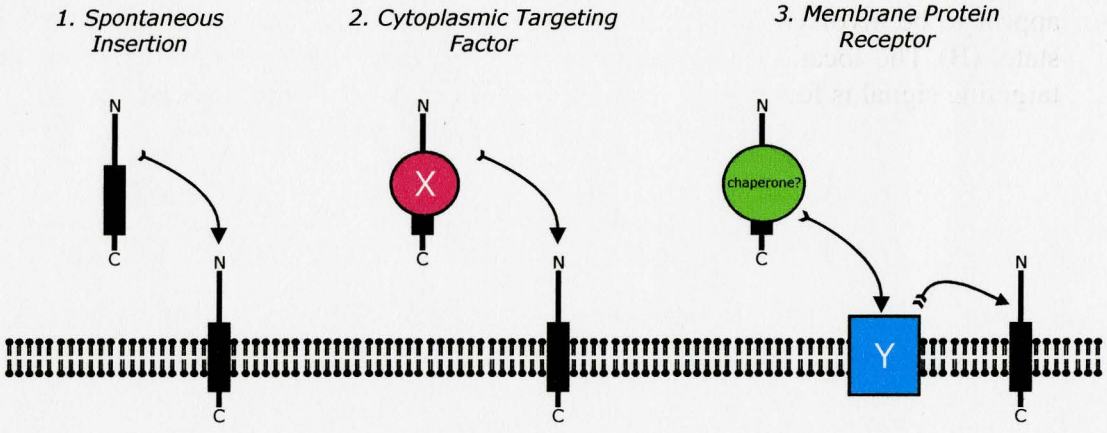
TA proteins are believed to be targeted via a post-translational, SRP-independent pathway (Anderson et al., 1983). There is controversial evidence suggesting that TA proteins are targeted to the ER independent of the translocon (Kutay et al., 1995; Walter et al., 2001, Steel et al., 2002; Yabal et al., 2002). The authors of one recent study contend that TA proteins transiently interact with translocon components during their insertion into the ER membrane (Abell et al., 2002). Nevertheless, current data points to three main targeting mechanisms for TA proteins, which are outlined in Figure 5. They are: (1) spontaneous insertion into the membrane, (2) targeting via a cytoplasmic factor, with spontaneous insertion into the membrane and (3) facilitated insertion into the membrane via a membrane bound receptor.

(1) Spontaneous insertion – In vitro targeting studies with the ER specific isoform of the mammalian TA protein cytochrome b_5 (Cb_5) have shown that it spontaneously integrates into liposomes (Anderson et al., 1983; Kim et al., 1997). Both Cb_5 and the proto-oncogene Bcl-2 insert into purified ER and mitochondria *in vitro*. Binding is non-saturable, ATP-independent, and does not require protease sensitive components on the membrane (Janiak et al., 1994; Kim et al., 1997). Although the data suggests that Bcl-2 will spontaneously insert into any membrane it encounters, unlike Cb_5, Bcl-2 does not efficiently integrate into liposomes (Janiak et al., 1994; Kim et al., 1997). Lipid composition or membrane structure may therefore be important for targeting since Bcl-2 can spontaneously insert into purified yeast mitochondria with almost 50% efficiency (Motz et al., 2002).

(2) Cytoplasmic targeting factors – Although Cb_5 will insert into any membrane presented to it *in vitro*, it is exclusively targeted to the ER *in vivo* (Mitoma & Ito, 1992). Our group partially reconstituted this specificity *in vitro*, as Cb_5 was found to preferentially target to the ER when both purified ER and mitochondria were present (Janiak et al., 1994). The specificity for ER membranes may be due to a preference for ER lipids, however the data does not rule out the potential role of cytoplasmic factors in targeting Cb_5. In support of this hypothesis, a recent study demonstrated that at least one cytoplasmic protein was required for the targeting of Cb_5 to the ER *in vitro* (Yabal et al., 2002). Further evidence for the role of cytoplasmic factors in TA protein targeting come from the study of peroxisomal ascorbate peroxidase (APX) in tobacco plants. APX was found to require molecular chaperones and ATP for efficient targeting (Mullen et al., 1999). Since neither APX nor Cb_5 require membrane associated proteins for targeting, molecular chaperones may target them through an as yet unknown mechanism. Once targeted, it is presumed that the proteins would spontaneously insert into the membrane. Clearly more research must be undertaken to confirm the involvement of cytoplasmic

Figure 5

A Mechanisms of Tail-Anchored Protein Targeting to Membranes



B Location of Tail-Anchor Protein Targeting Signals

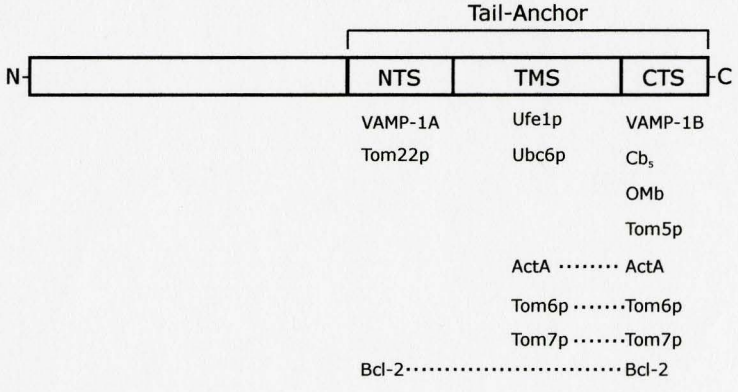


Figure 5: The targeting of Tail-Anchor (TA) proteins

(A) There are three proposed mechanisms for the targeting and insertion of TA proteins into membranes. (1) Spontaneous Insertion - involves no other proteins except the TA protein and is either kinetically or thermodynamically driven through the direct interaction of the TA with lipids. (2) Cytoplasmic Targeting Factor – a cytoplasmic protein (X) may bind to a TA protein and target it to the proper membrane. This factor may also keep the TA protein in an insertion competent state. Once targeted, insertion of the hydrophobic sequence of the TA is spontaneous. (3) Membrane Protein Receptor – a membrane protein (Y) may act as a receptor for the TAP, sequestering it at the proper membrane and facilitating its insertion into the membrane. Cytoplasmic chaperones appear to be involved in this process and likely keep the TAP in an insertion competent state. (B) The location of targeting signals for various TA proteins. In some cases the targeting signal is found in disparate parts of the TA – these are indicated by (·····).

factors in targeting, as the participation of chaperones may be limited to maintaining the proteins in an insertion competent state.

(3) Membrane associated targeting factors – Evidence of a role for membrane associated receptors in the targeting of TA proteins come from work on mammalian vesicle associated membrane proteins (VAMPs), the yeast vacuolar fusion protein Nyv1p, Bcl-2 and a splice isoform of Cb_5 termed outer mitochondrial membrane cytochrome b_5 (OMb). *In vitro* studies of the ER resident protein VAMP-1A have demonstrated that targeting is saturable, ATP-dependent and trypsin sensitive, thus implicating a membrane bound protein that is essential for targeting (Kutay et al., 1995; Kim et al., 1997; Kim et al., 1999). Although disputed, cross-linking and immunoprecipitation studies indicate that these proteins are members of the translocon (Abell et al., 2002). A mitochondrial resident splice isoform of VAMP-1A, called VAMP-1B (Isenmann et al., 1998), showed saturable, but ATP-independent targeting to purified mitochondria *in vitro*. In this system, ATP was only needed when molecular chaperones were present during the targeting reaction (Lan et al., 2000). The saturability of VAMP-1B targeting is indicative of a receptor on the mitochondria that recognizes VAMP-1B.

Nyv1p targeting to purified yeast ER was shown to be ATP-dependent and trypsin sensitive, yet contrary to the findings of Abell et al., the trypsin sensitive components were not members of the translocon (Steel et al., 2002). Bcl-2 targeting to purified yeast mitochondria has been shown to rely, in part, on the outer mitochondrial membrane protein import receptor Tom20p; however Bcl-2 is integrated into the membrane independent of the general import pore (Motz et al., 2002). OMb targets preferentially to mitochondria *in vivo*, even though it will insert into ER membranes *in vitro* (Borgese et al., 2001). This data is suggestive of a receptor for OMb on the mitochondria, however the trypsin sensitivity and saturation of OMb targeting must be demonstrated before the involvement of a membrane receptor can be concluded. Overall, the evidence indicates that some TA proteins are targeted via an interaction with a protein receptor on the membrane, and that cytoplasmic chaperones may play a role in their targeting, possibly by maintaining the TA protein in an insertion competent state.

1.4.2. Targeting signals of tail-anchor proteins

Just as there are multiple mechanisms for TA protein targeting, there too are multiple targeting signals within the protein sequence of different TA proteins. All targeting signals to date have been localized to the C-terminal TA region (Figure 5B). The TA consists of the N-terminal sequence (NTS), which encompasses the 10 to 30 amino acids immediately N-terminal to the TMS, the TMS itself, and the short stretch of amino acids immediately following the TMS, termed the C-terminal sequence (CTS). Besides anchoring the protein to a membrane, housing the targeting signal appears to be the sole role of the tail-anchor in TA proteins, as only one protein to date has demonstrated a functional role for the TA (Rayner & Pelham; 1997).

The targeting signal for VAMP-1A has been identified as a series of lysine residues that form an amphipathic alpha helix in the NTS (Kim et al., 1999). The TMS and CTS play no role in targeting or function, as they can be substituted entirely with a polyleucine tail (Whitley et al., 1996). However VAMP-1B, which is identical to VAMP-

1A except it carries a shortened TMS and a unique CTS that contains two positive amino acid residues, is targeted to mitochondria. The mitochondrial targeting signal is therefore located in the CTS (Isenmann et al., 1998) and is dominant to the ER signal in the NTS.

Many proteins that are part of the translocase of the outer mitochondrial membrane, called 'TOM' proteins, are TA proteins. The mitochondrial targeting information for Tom6p and Tom7p are found in the combined TMS and CTS, while the NTS is needed for assembly into the TOM complex (Dembowski et al., 2001). The NTS of Tom22p, and the positive residues in the CTS of Tom5p, are sufficient to target GFP to the mitochondria as long as they are situated next to a TMS (Egan et al., 1999; Horie et al., 2002). A recent study has also found that a conserved proline residue in the TMS of these TOM proteins is necessary for their targeting in yeast (Allen et al., 1997).

The TMS and CTS of the *Listeria monocytogenes* protein ActA together have been shown to be sufficient to target proteins to the mitochondria in mammalian cells (Zhu et al., 1996). The targeting signal for the ER t-snare Ufe1p, and the ER ubiquitin conjugating enzyme Ubc6p, have been localized to the TMS (Rayner & Pelham, 1997; Yang et al., 1997). The targeting signal for Cb_5 and OMb are located in the CTS (Mitoma & Ito, 1992; Kuroda et al., 1998). The mitochondrial targeting signal for Bcl-2 appears to consist of two single lysine residues – one in the NTS with the other in the CTS (Motz et al., 2002). The signals for Cb_5, OMb and Bcl-2 must be situated next to a TMS in order for targeting to occur. To date, no consensus ER targeting signal for TA proteins has been identified, while the mitochondrial targeting signal appears to be composed of loosely ordered basic amino acid residues in the CTS.

1.5. Summary of findings

We demonstrate that the TA protein Sss1p contains an ER targeting signal similar to mammalian VAMP-1A. Disruption of this signal causes incomplete mislocalization of Sss1p to the mitochondria that does not affect yeast growth. We show that mutations in the TMS and CTS of Sss1p diminish yeast growth, cause defects in co- and post-translational translocation and ribosome binding to Sec61p, and lead to the mislocalization of translocon components from light membranes (predominantly ER) to heavy membranes (predominantly mitochondria). Based on these results, we argue two possible functions for Sss1p: Sss1p is involved in forming the signal sequence binding pocket of the translocon and/or is essential for the integrity of the PCC.

2. Materials and Methods

2.1. Reagents

Iodoacetamide, PMSF, Yeast Nitrogen Base (without amino acids and with ammonium sulphate), and amino acids were purchased from Sigma. COMPLETE© protease inhibitor tablets were purchased from Roche. Zymolyase 100-T was purchased from ICN Biomedicals. Mouse anti-Actin monoclonal antibody, donkey anti-mouse HRP and donkey anti-rabbit HRP secondary antibodies were purchased from Jackson Laboratories. All Western blots were developed using Kodak Xomat-Blue film.

2.2. DNA Protocols

2.2.1. PCR from plasmid DNA

(all volumes in this table are in µL unless otherwise indicated)
(NOTE: Taq polymerase synthesizes 1-2 kb per minute)

	Reaction				No DNA negative control		
$[Mg^{2+}]$ (mM)	*0*	*2*	*4*	*8*	*2*	*4*	*8*
PCR Buffer (10X stock)	10	10	10	10	10	10	10
DNA (1/100 dilution)	1	1	1	1	0	0	0
Forward Primer (1/60)	5	5	5	5	5	5	5
Reverse Primer (1/60)	5	5	5	5	5	5	5
dNTPs (5mM stock)	5	5	5	5	5	5	5
$MgCl_2$ (25mM)	0	8	16	32	8	16	32
Taq-Vent [40:1 (v:v)]	1	1	1	1	1	1	1
dH_20	73	65	57	41	66	58	42

Reaction Conditions:			
	1 cycle	94°C	5 min
	35 cycles	94°C	30 sec
	Annealing Temperature		30 sec
		72°C	2 min
	1 cycle	72°C	7 min
	end	4°C	

Suspend 5 µL of each tube in 10 µL DNA Loading Dye and run on a 1% agarose gel to visualize amplified PCR product.

2.2.2. DNA purification from agarose gels

(This protocol is useful for purifying DNA fragments larger than 500 bp)

1. Add 300 µL NaI per 100 mg gel slice.
2. Heat at 55°C until dissolved (vortex periodically).
3. Add 3 µL glassmilk beads (vortex briefly).
4. Incubate at 55°C for 10 minutes (vortex briefly every 2 minutes). DNA will bind to beads.
5. Spin for 15 seconds at maximum speed in a tabletop centrifuge at RT to pellet beads.
6. Aspirate the supernatant.
7. Wash 3X with 500 µL New Wash.

8. Elute DNA from beads with 20 µL TE buffer at 55°C for 10 minutes (gently resuspend beads every 3 minutes).
9. Spin at maximum speed for 1 minute to pellet beads. Remove 20 µL TE and place into a clean eppendorf tube, discarding the beads. Spin new tube at maximum speed for 1 minute. Remove 15 µL and place in a clean eppendorf tube (this spin removes residual beads). Add 10 µL of DNA loading dye to the 5 µL of TE left over in the old tube. Run this sample on a 1% agarose gel and visualize to verify that the DNA product was successfully purified.
10. Purified DNA can be stored at -20°C.

TE Buffer
50 mM Tris·HCl pH 8
20 mM EDTA pH 8

2.2.3. Isopropanol precipitation of DNA

(great for PCR products)

1. Add 2 volumes of 100% isopropanol.
2. Vortex at maximum for 20 seconds.
3. Spin at maximum speed for 10 minutes in a tabletop centrifuge to pellet DNA. Aspirate the supernatant.
4. Wash DNA pellet 1X with 500 µL 70% ethanol.
5. Spin at maximum speed for 10 minutes in a tabletop centrifuge to pellet DNA. Aspirate the supernatant.
6. Dry the DNA pellet in a speed-vac for 5 minutes.
7. Dissolve DNA pellet in 20 µL TE buffer.

TE Buffer
50 mM Tris·HCl pH 8
20 mM EDTA pH 8

2.2.4. Phosphorylation, annealing and ligation of cassette oligonucleotides

(A) Phosphorylation of oligonucleotides

In a clean eppendorf tube add:
1 µL oligonucleotide (300 pmol/µL)
1 µL PNK (PolyNucleotide Kinase)
1.4 µL PNK buffer (10X stock)
1 µL ATP (10mM)
9.6 µL dH_20

Incubate this tube for 1 hour at 37°C. Heat inactivate PNK at 65°C for 20 minutes.

(B) Annealing Reaction

In a clean eppendorf tube add:
3 µL phosphorylated oligo #1
3 µL phosphorylated oligo #2
3 µL Annealing Buffer (10X stock)
21 µL dH_20

Incubate this tube at 95°C for 10 minutes, then remove the block from the heating apparatus and allow to cool to 30°C.

10X Annealing Buffer
200 mM Tris·HCl pH 8
100 mM $MgCl_2$
500 mM NaCl
10 mM DTT

(C) Ligation Reaction

(all volumes in this table are in µL unless otherwise indicated)
Note: 'Annealed Oligo' is a 1/10 dilution of the Annealing Reaction

	negative control	*1:1*	*2:1*	*3:1*
Vector	0.3	0.3	0.3	0.3
Annealed Oligo (1/10)	0	0.3	0.6	1
Ligase Buffer (10X)	1	1	1	1
T4 DNA Ligase	1	1	1	1
ATP (10 mM)	0.25	0.25	0.25	0.25
EDTA (2.5 mM)	1	1	1	1
dH_20	6.45	6.15	5.85	5.45

Ligations times will vary – try 1 hr at RT, 4 hr at RT, and O/N at 16°C.

2.3. Plasmid construction

Plasmid backbones:
pMAC1148 (pRS313) – yeast low-copy shuttle vector
pMAC1376 (pRS423) – yeast high-copy shuttle vector
pMAC651 (pSPUTK) – vector for expression of proteins in eukaryotic cell free lysates.

Wt Sss1p (pMAC1150 - made by Nawaid Usmani): Primers of the following sequence 5`-GTGACCGTCCTAGACCTCAG-3` and 5`-GGGCTCTAGACATGATCGGTATGGAGCTTA-3`, containing a 5` SalI and XbaI restriction site respectively, were used to amplify the *SSS1* coding sequence, along with the endogenous promotor and 3`UTR, from purified yeast genomic DNA. Using these restriction sites, the PCR product was cloned into the low-copy yeast shuttle vector pRS313 (named 1150). For high copy expression of Sss1p, the *SSS1* promotor, coding

sequence and 3`UTR were subcloned from pMAC1150 into the yeast shuttle vector pRS423, using ApaI and SacI restriction sites (1491).

Sss1pTom5 mutant (pMAC1395 - made by Hendrik Nieuwland): Primers of the following sequence 5`-CGTCCATGGTGCATGGCAATCAGCGAAGTA-3` and 5`-ATTCTCGAGTTATTTCCATTGCTTTTTCAC-3`, containing a 5` NcoI and XhoI restriction site respectively, were used to amplify the *TOM5* coding sequence, along with the 5`UTR, from purified yeast genomic DNA. Using these restriction sites, the PCR product was cloned into the plasmid pSPUTK, placing the *TOM5* sequence in front of the Sss1p 3`UTR, which had been cloned into pSPUTK. From this construct, primers of the following sequence 5`-GACCCATGGCTTAAGCAGGCCGCTTATGTGGCTG-3` and 5`-TCTACATGTGGGCCCTCTTTCCTGCGTTATCCCCT-3`, containing a 5` AflII and ApaI site respectively, were used to amplify the *TOM5* TMS, CTS and the Sss1p 3`UTR. Using these restriction sites (the AflII site had been previously placed in front of the TMS of Sss1p by site-directed mutagenesis), the PCR product was cloned into the low-copy yeast shuttle vector pRS313, so that the endogenous TMS and CTS of Sss1p was replaced by the TMS and CTS of Tom5p.

Sss1pActA mutant (pMAC1401 - made by Johnny Tkach and Hendrik Nieuwland): An AflII restriction site had been previously placed in front of the TMS of both ActA and Sss1p by site directed mutagenesis in the vector pSPUTK. Using the BglII and AflII restriction sites, the coding sequence of Sss1p (lacking the TMS, CTS and 3`UTR) was subcloned in front of the TMS of ActA. Using BamHI and XhoI restriction sites, the Sss1pActA coding sequence, as well as the endogenous *SSS1* promotor, was subcloned into the low-copy yeast shuttle vector pRS313 (pMAC1272). To place the Sss1p 3`UTR after the Sss1pActA coding sequence, the restriction sites XhoI and BsrGI were used subclone the Sss1pActA coding sequence from pMAC1272 in front of the 3`UTR from pMAC1395.

Sss1pCTSa mutant (pMAC1402 - made by Johnny Tkach and Hendrik Nieuwland): Using site directed mutagenesis, a KpnI site was inserted between the TMS and CTS of Sss1p, and between the TMS and CTS of ActA. The CTS of Sss1p was replaced with the CTS of ActA by using the restriction sites KpnI and NheI. To place the ORF back into frame, the KpnI site was removed by the Klenow enzyme. Using the restriction sites SexAI and XhoI, the Sss1pCTSa coding sequence, as well as the endogenous *SSS1* promotor, was subcloned into the low-copy yeast shuttle vector pRS313 (pMAC1381). To place the Sss1p 3`UTR after the Sss1pCTSa coding sequence, the restriction sites XhoI and BsrGI were used subclone the Sss1pActA coding sequence from 1381 in front of the 3`UTR from pMAC1395.

Sss1pTMSa mutant (pMAC1459 - made by Johnny Tkach and Hendrik Nieuwland): Using site directed mutagenesis, a KpnI site was inserted between the TMS and CTS of Sss1p, and between the TMS and CTS of ActA. The TMS of Sss1p was replaced with the TMS of ActA by using the restriction sites KpnI and NheI. To place the ORF back into

frame, the KpnI site was removed by the Klenow enzyme. The following set of primers were used to add an XhoI after the CTS of Sss1p, and an ApaI site at the end of the TMS of ActA in this mutant: (XhoI site) 5`-GAACTCGAGAAGAGATAAAAGACGAGAAAA-3`, 5`-ATTCTCGAGTTAAACAATAACGTATCTGA-3`, (ApaI site) 5`-TCGGGGCCCTCAAGTTGATTCAT-3`, 5`-TGAGGGCCCCGAGGGAGAACAC-3`. The Sss1pTMSa coding sequence, along with the 3`UTR of Sss1p, were subcloned into the low-copy yeast shuttle vector pRS313 using the restriction sites AflII and XbaI.

Sss1pCTSwtVIV/AAA mutant (pMAC1503 - made by Paulina Dlugosz): Using cassette mutagenesis, the last three amino acids of the CTS from Sss1p (V-I-V) were deleted and replaced with alanine residues. Two oligonucleotides (see chart below for nucleotide sequence) encoding the Sss1p CTS with the last three amino acids replaced by alanine residues were synthesized to form a cassette with ApaI and XhoI restriction sites at the 5` and 3` ends respectively. Using the restriction enzymes ApaI and XhoI, the cassette was cloned into the Sss1pTMSa mutant (pMAC1459), replacing the wild-type CTS with the mutant CTS.

Name (plasmid #)	Oligonucleotide sequence (two oligonucleotides form one cassette)
Sss1pCTSwtΔKLI (pMAC1507)	5` CTCGCCGCCGCCCATATTCCAATCAGATACGTAATTGTTTAAC 3` 5` TCGAGTTAAACAATTACGTATCTGATTGGAATATGGGCGGCGGCGAGGGCC 3`
Sss1pCTSwtKLI/AAA (pMAC1508)	5` CTCCATATTCCAATCAGATACGTAATTGTTTAAC 3` 5` TCGAGTTAAACAATTACGTATCTGATTGGAATATGGAGGGCC 3`
Sss1pCTSwtΔHIP (pMAC1471)	5` CTCAAATTAATTATCAGATACGTTATTGTTTAAC 3` 5`TCGAGTTAAACAATAACGTATCTGATAATTAATTTGAGGGCC 3`
Sss1pCTSwtHIP/AAA (pMAC1506)	5` CTCAAATTAATTGCCGCCGCCATCAGATACGTTATTGTTTAAC 3` 5` TCGAGTTTAACAATAACGTATCTGATGGCGGCGGCAATTAATTTGAGGGCC 3`
Sss1pCTSwtΔIRY (pMAC1504)	5` CTCAAATTAATTCATATTCCAGTTATTGTTTAAC 3` 5` TCGAGTTAAACAATAACTGGAATATGAATTAATTTGAGGGCC 3`
Sss1pCTSwtIRY/AAA (pMAC1505)	5` CTCAAATTAATTCATATTCCAGCCGCCGCCGTTATTGTTTAAC 3` 5` TCGAGTTAAACAATAACGGCGGCGGCTGGAATATGAATTAATTTGAGGGCC 3`
Sss1pCTSwtΔVIV (pMAC1440)	*see below
Sss1pCTSwtVIV/AAA (pMAC1503)	5` CTCAAATTAATTCATATTCCAATCAGATACGCCGCCGCCTAAC 3` 5` TCGAGTTAGGCGGCGGCGTATCTGATTGGAATATGAATTAATTTGAGGGCC 3`
Sss1pCTSwtGGG (pMAC1501)	5` CTCAAATTAATTCATATTCCAATCAGATACGTTATTGTTGGGGGGGGGTAAC 3` 5` TCGAGTTACCCCCCCCCAACAATAACGTATCTGATTGGAATATGAATTAAATTGAGGGCC3`

*The Sss1pCTSwtΔVIV mutant was constructed differently. Primers of the following sequence 5` TTAGAATTCTTAGTATCTGATTGGAATATGAATC 3` and 5` TGCGAATTCAAGAGATAAAAGACGAGAAA 3`, containing the restriction site EcoRI, were used to delete the last three amino acids of the CTS (V-I-V) by whole plasmid PCR. The mutant coding sequence was subcloned into the low-copy yeast shuttle vector pRS313 using the restriction enzymes AflII and XbaI.

Sss1pK5/T5 mutant (pMAC1537 - made by Hendrik Nieuwland): The *SSS1* promotor, coding sequence and 3` UTR was subcloned into the vector pSPUTK using the restriction enzymes ApaI and SacI. The restriction sites AflII and MfeI added were N-terminus and C-terminus of the NTS by whole plasmid PCR using the following primers: (AflII) 5` CGCCTTAAGCAAGTGTAAGAAACCTGATTTG 3` and 5` CGCCTTAAGAATTGAGTACCTTCTCTGACAAATTC 3`; (MfeI) 5` GCGTGTCAATTGGTATTGGTTTTATTGCAGTCGGTAT 3` and 5` CGGTCTCAATTGCCTTGACAATCTTGGTGTATTC 3`. The *SSS1* promotor, coding sequence containing the new restriction sites and 3` UTR were subcloned into the low-copy yeast shuttle vector pRS313 using the restriction enzymes ApaI and SacI (pMAC1510). Using cassette mutagenesis, the five lysine residues in the NTS were mutated to threonine residues. Two oligonucleotides of the following sequence: 5` TTAAGCACCTGTAAGACCCCAGATCTGACCGAATACACCACCATTGTCACCG C 3` and 5` AATTGCGGTGACAATGGTGGTGTATTCGGTCAGATCTGGGGTCTTACAGGTGC 3`, were synthesized to form a cassette with AflII and MfeI restriction sites at the 5` and 3` end respectively. Using the restriction enzymes AflII and MfeI, the cassette was cloned into pMAC1510, replacing the Wt NTS with the mutant NTS.

2.4. Manipulation of yeast

2.4.1. Yeast genotypes and phenotypes

This is a brief description of the nomenclature used in yeast genetics.

Genotype	Phenotype
ade2-1	Defect in the *ADE2* gene. Auxotrophic for adenine.
can1-100	Canavanine (analogue of arginine) resistance
his3-11,-15	Defect in the *HIS3* gene. Auxotrophic for histidine.
leu2-3,-112	Defect in the *LEU2* gene. Auxotrophic for adenine.
trp1-1	Defect in the *TRP1* gene. Auxotrophic for tryptophan.
ura3-1	Defect in the *URA3* gene. Auxotrophic for uracil.
MATa	a mating type. Synthesizes mating factor a.
MATα	α mating type. Synthesizes mating factor α
sss1::URA3	Indicates that the genomic copy of the *SSS1* gene has been knocked out by the *URA3* gene.
p[*GAL10::SSS1, ADE2*]	Indicates that the yeast carry a plasmid that has the *SSS1* gene under the *GAL10* promotor. This plasmid carries the *ADE2* gene.

2.4.2. Yeast growth medium

2.4.2.1. Yeast Extract Peptone (YEP) medium

Yeast extract peptone glucose (YEPD) or galactose (YEPG) medium is used for routine growth of yeast. It is prepared by adding yeast extract, peptone and agar (if needed) in the desired volume of distilled water. The pH is adjusted to 6.5 using acetic acid. After solutions have been autoclaved, the filter-sterilized carbon source

(glucose/galactose) can be added to make up the appropriate medium. The recipe for 1 L of medium is below.

For 1 L of YEP Medium

Recipe	YEPD	YEPG
1% Yeast Extract	10 g	10 g
1% Peptone	10 g	10 g
2% Agar (for plates only)	20 g	20 g
Distilled Water	Up to 900 mL	Up to 900 mL
2% Carbon Source	100 mL of 20% glucose	100 mL of 20% galactose

2.4.2.2. Drop-Out Yeast Extract Peptone (D.O. YEP) Medium

D.O. medium is used to select for auxotrophic yeast that carry a gene (be it on a plasmid or a genomic integration) linked to a prototrophic genetic marker. For example, some yeast are tryptophan auxotrophs. For these yeast to survive, tryptophan must be supplied in the growth medium, since the yeast contain a genetic defect in the TRP1 gene that is responsible for the synthesis of tryptophan. In a similar fashion as ampicillin is used to select for bacteria carrying plasmids with the beta-lactamase gene, tryptophan can be 'dropped-out' of the growth medium to select for yeast which carry the TRP1 gene.

To prepare D.O. medium, begin by dissolving the D.O. Mix (see below) in a small volume of distilled water (i.e. 100 mL for every 1 L of D.O. medium to be made). Use concentrated HCl to bring the pH to 1.0, or until all of the amino acids have dissolved. Add this solution to a larger beaker containing Yeast Nitrogen Base (YNB - without amino acids and with ammonium sulphate) in distilled water (i.e. 650 mL for every 1 L of D.O. medium to be made) and allow this solution to stir for 10-15 minutes. Use concentrate NaOH pellets to bring the pH up to 6.5 and make up the desired final volume with distilled water (i.e. 900 mL final volume for 1 L of D.O. medium). After autoclaving this solution, add the desired carbon source (glucose or galactose) and any amino acid solutions necessary (tryptophan or histidine, both which cannot be autoclaved). The recipe for 1 L of D.O. medium is below.

For 1 L D.O. Medium

Recipe	D.O. YEPD	D.O. YEPG
0.67% YNB (without amino acids) (with ammonium sulphate)	6.7 g	6.7 g
0.2% Drop-Out Mix	2 g	2 g
2% Bacto-agar (for plates only)	20 g	20 g
Distilled Water	Up to 900 mL	Up to 900 mL
2% Carbon Source (filter sterilized)	100 mL 20% glucose	100 mL 20% galactose
Tryptophan or Histidine (filter sterilized)	12 mL of 1% solution	12 mL of 1% solution

2.4.2.3. Drop-Out (D.O.) mix (-Histidine, -Tryptophan)
Add the following amounts to a mortar and pestle and grind into a fine powder.

Adenine	0.5 g	Alanine	2.0 g
Arginine	2.0 g	Aspartic Acid	2.0 g
Cysteine	2.0 g	Glutamine	2.0 g
Glutamic Acid	2.0 g	Glycine	2.0 g
Inositol	2.0 g	Isoleucine	2.0 g
Leucine	4.0 g	Lysine	2.0 g
Methionine	2.0 g	Para-amino benzoic acid	0.2 g
Phenylalanine	2.0 g	Proline	2.0 g
Serine	2.0 g	Threonine	2.0 g
Tyrosine	2.0 g	Uracil	2.0 g
Valine	2.0 g		

2.4.3. Yeast protocols

2.4.3.1. Rapid isolation of yeast chromosomal DNA

1. Seed 10 mL of D.O. YEPG medium with a saturated yeast culture and grow O/N at 30°C.
2. Spin culture for 5 minutes at 5000 rpm (4000*xg*) in a Beckman JA-20 rotor at RT (All subsequent spins will be at RT). Aspirate the supernatant and resuspend cells in 500 µL dH_20.
3. Transfer the resuspended cells to an eppendorf tube and spin for 5 seconds at maximum speed in a tabletop centrifuge. Aspirate the supernatant and disrupt pellet by vortexing briefly.
4. Resuspend cells in 200 µL Breaking Buffer. Add 0.3 g glass beads (~200 µL volume), 200 µL Phenol/Chloroform (50:50) and vortex at highest speed for 3 minutes.
5. Add 200 µL TE buffer and vortex briefly.
6. Spin for 5 minutes at maximum speed and transfer the aqueous layer to a clean eppendorf tube. Add 1 mL of 100% ethanol and mix by inversion.
7. Spin for 3 minutes at maximum speed. Aspirate supernatant and air-dry the pellet.
8. Resuspend the pellet in 400 µL TE Buffer+1mg/mL RNase buffer and incubate for 5 minutes at 37°C.
9. Add 10 µL of 4 M ammonium acetate and 1 mL of 100% ethanol. Mix by inversion.
10. Spin 3 minutes at maximum speed. Aspirate the supernatant and allow pellet to air dry. Resuspend chromosomal DNA pellet in 100 µL TE buffer.

Breaking Buffer
2% TX-100
1% SDS
100 mM NaCl
10 mM Tris·HCl pH 8
1 mM EDTA pH 8

TE Buffer
50 mM Tris·HCl pH 8
20 mM EDTA pH 8

2.4.3.2. PCR from yeast genome

(all volumes in this table are in µL unless otherwise indicated)

	Reaction				No DNA negative control		
$[Mg^{2+}]$ (mM)	0	2	5	10	2	5	10
PCR Buffer (10X stock)	10	10	10	10	10	10	10
DNA (1/10 dilution)	5	5	5	5	0	0	0
Forward Primer (1/50)	5	5	5	5	5	5	5
Reverse Primer (1/50)	5	5	5	5	5	5	5
dNTPs (5mM)	5	5	5	5	5	5	5
$MgCl_2$ (25mM)	0	8	20	40	8	20	40
Taq-Vent [20:1 (v:v)]	1	1	1	1	1	1	1
dH_20	68	60	48	28	65	53	33

Reaction Conditions:			
	1 cycle	94°C	5 min
	35 cycles	94°C	30 sec
		Annealing Temperature	30 sec
		72°C	2 min
	1 cycle	72°C	7 min
	end	4°C	

Suspend 5 µL of each tube in 10 µL DNA Loading Dye and run on a 1% agarose gel to visualize amplified PCR product.

2.4.3.3. Transformation of yeast

1. Inoculate 2 mL of D.O. YEPG with a yeast colony and grow overnight (O/N) to saturation at 30°C in a rotator.
2. Take 500 µL of culture and spin for 5 minutes at 1,000*xg* in a tabletop centrifuge at room temperature. Aspirate the supernatant but USE CAUTION - yeast pellets are easily sucked up by aspiration.
3. Add 10 µL of salmon sperm DNA (10 mg/mL - boiled at 90°C for 10 minutes) and 1 µg of transforming DNA. Vortex the mixture.
4. Add 500 µL Transformation Buffer.
5. Incubate O/N at RT.
6. Spread 100-300 µL of cells (from the bottom of the tube) directly onto D.O. YEPG plates.

Transformation Buffer
90 mL sterile 45% PEG 4000
10 mL 1 M lithium acetate
500 µL 2 M Tris·HCl pH 7.5
200 µL 0.5 M EDTA pH 8

2.4.3.4. Yeast growth assay

This assay is designed to test the ability of plasmid expressed Sss1p mutant proteins to complement a growth defect under non-permissive growth conditions (YEPD) where endogenous wild-type Sss1p expression is repressed. This assay can also determine

if the Sss1p mutant proteins are dominant or recessive by growing yeast under permissive conditions (YEPG) where both the mutant Sss1p and Wt Sss1p proteins are expressed.
NOTE: For yeast strains: 1 $O.D._{600}$/mL ~ $2.0x10^7$ cells per mL.

1. Inoculate 2-5 mL of Drop-Out (D.O.) YEPG medium with the yeast under study and grow to saturation at 30°C in a rotator overnight.
2. Pellet 1 $O.D._{600}$ unit of cells in a microcentrifuge and aspirate supernatant. Suspend pellet in 1mL dH_2O to create a 1 $O.D._{600}$/mL culture tube.
3. Do four 1/10 serial dilutions (take 100 µL of 1 $0.D._{600}$/mL tube and add to 900 µL dH_2O to make a 0.1 OD_{600}/mL tube, then proceed using the 0.1 $O.D._{600}$/mL tube.) You should end up with five tubes ranging from 1 OD_{600}/mL to 0.0001 OD_{600}/mL.
4. Spot 5 µL of each tube in a row on a permissive (D.O. YEPG – Wt Sss1p expressed) and non-permissive (D.O. YEPD – Wt Sss1p repressed) selective medium plate, starting with the 1 $O.D._{600}$/mL culture tube (each successive spot should contain 10^5, 10^4, 10^3, 10^2, 10 cells).
5. Incubate plates at 30°C for 2-3 days. Heat or cold sensitivity can be tested by incubating plates at 37°C for 2-3 days or 17°C for 7 days.

2.4.3.5. Analysis of protein expression in yeast

1. Inoculate 2-5 mL of Drop-Out (D.O.) YEPG liquid medium with a yeast colony and grow to saturation at 30°C in a rotator overnight.
2. Suspend culture to 0.10 - 0.15 $O.D._{600}$/mL in 5 mL D.O. YEPD liquid medium and grow in a rotator at 30°C for a minimum of 8 hours. After 8 hours GAL10::*SSS1* expression is repressed and endogenous Wt Sss1p protein is completely degraded while plasmid borne mutant Sss1p protein is expressed under its endogenous promotor. Therefore the only protein present in the cell will be mutant Sss1p.
3. Ensure that the cells are in log phase (0.5-5 $O.D._{600}$/mL). All strains should be in early log phase after 8 hours (between 0.5-1 $O.D._{600}$/mL).

Prepare protein samples as outlined in "Yeast whole cell lysates for SDS-PAGE".

2.4.3.6. Yeast whole cell lysates for SDS-PAGE

1. Pellet 1 $O.D._{600}$ unit of yeast cells in an eppendorf tube.
2. Add 40 µL TLB.
3. Boil immediately for 2 minutes (For membrane proteins, heat to 65°C for 10 min).
4. Add ~0.12 g glass beads. (NOTE: scoops can be made to allow rapid measurement of 0.12 g glass beads by cutting off the bottom of an eppendorf tube.) Glass beads are prepared by washing in concentrated nitric acid overnight, rinsing thoroughly with water and baking.
5. Vortex at highest speed for 1 minute to disrupt yeast cells.
6. Add 160 µL TLB and vortex. Heat samples again.

7. Spin samples at maximum speed in a table-top centrifuge for 1 minute to pellet beads. Collect 150 µL of sample and place in a clean eppendorf tube. Final concentration will be $1x10^5$ cells/µL.
8. Samples can be stored at –20°C.
9. Briefly vortex and boil the cell extracts prior to loading the gel (For membrane proteins, heat to 65°C for 10 minutes).

TLB
80 mM TrisHCl pH 6.8
20% SDS
0.1% bromophenol blue
100 mM DTT
10% glycerol

2.4.3.7. Western blot antibody dilutions

1° Antibody	Animal	Dilution	2° Dilution	Source
Sss1p	rabbit polyclonal	1:5,000	1:25,000	This study – Johnny Tkach
Sec61p	rabbit polyclonal	1:30,000	1:60,000	Dr. Jeff Brodsky
Sec62p	rabbit polyclonal	1:5,000	1:60,000	Dr. Jeff Brodsky
Sec63p	rabbit polyclonal	1:10,000	1:60,000	Dr. Randy Schekman
Kar2p	rabbit polyclonal	1:60,000	1:60,000	Dr. Jeff Brodsky
ppαF	rabbit polyclonal	1:5,000	1:30,000	Dr. Randy Schekman
Mir1p	rabbit polyclonal	1:100,000	1:60,000	Dr. Robert Jensen
L3	mouse monoclonal	1:10,000	1:60,000	Dr. Jonathon Warner
Actin	mouse monoclonal	1:80,000	1:60,000	commercial

2.4.3.8. Purification of yeast endoplasmic reticulum
Adapted from Meisinger et al., 2000 (see Bibliography)

1. Grow a 5 mL culture of yeast in Drop-Out (D.O.) YEPG liquid medium to saturation at 30°C overnight (O/N).
2. Add saturated culture to 50 mL D.O. YEPG liquid medium and grow at 30°C O/N.
3. Suspend cells to 0.10 - 0.15 OD_{600}/mL in 2 L D.O. YEPD liquid medium. Grow for 8 hours at 30°C (to shut down endogenous Sss1p expression). Culture should be at an OD_{600}/mL between 0.5 – 2.0.
4. Pellet cells at 5000 rpm in a JLA 9.1 rotor at 4°C for 5 minutes.
5. Wash 1X with 500 mL distilled water.
6. Suspend cells in 20 mL Spheroplast Buffer (use 1 complete protease pellet/45 mL buffer).
7. Convert cells to spheroplasts by adding 0.1 mg/mL Zymolyase 100T (1 mL of 2 mg/mL stock) and incubate at 30°C with gentle agitation for 1 hour (100 rpm).

****From this point on, all solutions are ice-cold, and all spins are done at 4°C.****

8. Pellet spheroplasts by centrifugation at 3,600 rpm (2,300x*g*) for 8 minutes in an SW28 rotor.
9. Resuspend spheroplasts in 18 mL SKEEM buffer (use 1 complete protease pellet/40 mL buffer). Add PMSF and iodoacetamide to 1 mM.

10. Transfer resuspended spheroplasts to a Dounce homogenization tube and disrupt by 20 strokes of a Dounce pestle.
11. Centrifuge homogenate at 2,400 rpm (1,000x*g*) for 10 minutes in an SW28 rotor to yield a post-nuclear supernatant (PNS) and nuclear pellet (NP). The NP can be discarded.
12. Centrifuge the PNS at 11,000 rpm (20,000x*g*) for 30 minutes in an SW41 rotor to yield pellet (20KgP – heavy membranes) and supernatant (20KgS – light membranes) fractions. The 20KgP can be discarded.
13. Split the 20KgS in half (one half for RM and the other half for PKRM). Centrifuge at 29,500 rpm (150,000x*g*) for 30 minutes in an SW41 rotor to yield pellet (100KgP) [purified rough microsomes (RM)] and supernatant (100KgS) [cytosol].
    Suspend RM in TSM buffer (add 1X PIN and 1mM PMSF) with a glass-teflon homogenizer. Determine protein equivalents by diluting RM 1:200 in 2% SDS and measuring absorbance at 280 nm (A_{280}) [Remember to measure the A_{280} of the 2% SDS and compare it to a dH_2O blank!]. Suspend microsomes to 25 A_{280} units/mL in TSM. 50 – 200 µL aliquots can be snap frozen in liquid N_2 and stored at –80°C.
14. To make ribosome stripped rough microsomes (PKRM), suspend 100KgP in STRIP buffer. Add puromycin to 1 mM, vortex and place on ice for 30 minutes. Layer membranes over a 0.5 M sucrose cushion (made in STRIP buffer) and centrifuge at 48,000 rpm (100,000xg) for 30 minutes in a TLA 100.2 rotor.
    Suspend pellet in TS Buffer (add 1X PIN and 1 mM PMSF) with a pipette. Determine protein equivalents by absorbance at 280 nm as described in Step 13. Suspend microsomes to 25 A_{280} units/mL. 50 – 100 µL aliquots can be snap frozen in liquid N_2 and stored at –80°C.

Spheroplast Buffer
5 mM MOPS·KOH pH 7.2
0.5 M KCl
10 mM sodium sulphite (activates Zymolyase 100T)

TSM Buffer
20 mM Tris·HCl pH 7.4
5 mM $Mg(OAc)_2$
250 mM sucrose

SKEEM Buffer
5 mM MES·KOH pH 5.5
1 M sorbitol
0.5 mM EDTA
1 mM KCl
0.1% ethanol

STRIP Buffer
20 mM Tris·HCl pH 7.4
50 mM EDTA
500 mM K0Ac

TS Buffer
20 mM Tris·HCl pH 7.4
250 mM sucrose

0.5 M Sucrose Cushion in STRIP
(for 10 mL)
0.86 g (since STRIP already has 250 mM sucrose)

2.4.3.9. Fractionation of yeast heavy membranes, light membranes and cytosol

1. Inoculate a 50 mL culture of D.O. YEPD to 0.10 - 0.15 OD_{600}/mL and grow for 12 hours at 30°C (to an OD_{600}/mL between 1 and 5).
2. Pellet 40 OD_{600} units of cells ($8x10^8$ cells) in a clinical centrifuge (setting #3) for 10 minutes at 4°C. Suspend cells in 910 µL Spheroplast buffer plus 40 µL 25X complete protease inhibitor cocktail (CPIC). Take 25 µL cells in 75 µL TLB, add

glass beads and vortex to generate yeast whole cell lysates – final concentration is $2x10^5$ cells/μL.

3. Spheroplast cells with 0.1 mg/mL Zymolyase 100T (50 μL of a 2 mg/mL stock) at 30°C for 30 minutes (with an end-over-end rotator).

NOTE: All subsequent steps to be performed at 4°C.

4. Pellet spheroplasts at 1,000*xg* (3,300 rpm) for 5 minutes in a tabletop centrifuge.
5. Suspend spheroplasts in 1.88 mL SKEEM buffer, 80 μL 25X CPIC, 20 μL 100 mM PMSF and 20 μL 100 mM iodoacetamide. Break spheroplasts by 20 strokes in a Dounce homogenizer.
6. Spin homogenate at 2.1K*xg* (5K rpm) for 3 minutes in a TLS 55 rotor to pellet nuclei and cell debris, leaving a post-nuclear supernatant (PNS). Transfer PNS to a new TLS 55 tube.
7. Spin PNS at 20K*xg* (15K rpm) for 10 minutes in a TLS 55 rotor, to generate a pellet (20KgP - heavy membranes (HM)] and supernatant [20KgS - light membranes (LM)]. Transfer the 20KgS to a new TLS 55 tube. Suspend the 20KgP in 200 μL SEM buffer (plus 1 mM PMSF, 1X PIN), layer over a 1.7 mL 60% sucrose cushion (in EM buffer) and spin at 150K*xg* (42K rpm) for 30 minutes (to separate aggregate matter from the HM). Collect 300 μL from the top of the tube (HM fraction) and TCA precipitate. Suspend HM pellet in 100 μL hot TLB. (This makes the HM fraction 40X the WCL, or $8x10^6$ cells/μL).
8. Spin the 20KgS at 150K*xg* (42K rpm) at for 30 minutes in a TLS 55 rotor, to generate 150KgP (LM) and 150KgS (cytosol) fractions. Suspend the LM in 100 μL hot TLB. TCA precipitate the cytosol and suspend in 100 μL hot TLB. (This makes the LM and cytosol fractions 40X the WCL, or $8x10^6$ cells/μL.)

25X CPIC – 1 pellet in 2 mL 100 mM KPO_4 pH 6.8
100 mM iodoacetamide – 18.5 mg/mL in water
100 mM PMSF – 17 mg/mL in 100% ethanol

SEM buffer
250 mM sucrose
5 mM MOPS·KOH pH 7.2
1 mM EDTA

EM buffer
5 mM MOPS·KOH pH 7.2
1 mM EDTA

2.4.3.10. Integral/peripheral membrane protein assay

Adapted from Esnault et al., 1994 (see Bibliography)

1. Subject 10 - 20 μL (250 – 500 A_{280} μUnits) of RMs (add 1 μL 200X PIN and 1 μL 100 mM PMSF) to the following treatments:
    - 78-88 μL TSM buffer at 24°C for 60 minutes.
    - 78-88 μL 2.5 M urea (in TSM) at 24°C for 60 minutes.
    - 78-88 μL 0.5 M KOAc (in TSM) at 0°C for 30 minutes.
    - 78-88 μL 0.1 M carbonate pH 11.5 at 0°C for 30 minutes with 6X vortexing at maximum for 10 seconds during the incubation.
2. Layer the samples over a 50 μL 0.5 M sucrose cushion (made in the same buffer) and spin at 50K rpm (100K*xg*) at 4°C in a TLA 100 rotor for 20 minutes to yield a

100KgS (peripheral membrane proteins) and 100KgP (integral membrane proteins).

3. TCA precipitate the 100KgS and suspend in 50 µL hot TLB. Suspend the 100KgP in 50 µL hot TLB.
4. Run 15 µL per lane for Western analysis.

TSM Buffer
20 mM Tris·HCl pH 7.4
5 mM $Mg(OAc)_2$
250 mM sucrose

2.4.3.11. Assay for ribosome associated membrane proteins (RAMPs)

Adapted from Kalies et al., 1994 and Gorlich & Rapoport, 1993 (see Bibliography).

1. Add the following components to an eppendorf tube
   - 10-20 µL membranes (250-500 A_{280} µUnits)
   - 1 µL 200X PIN
   - 1 µL 100 mM PMSF
   - 1 µL 100 mM $CaCl_2$
   - 0.5 µL 10 U/µL micrococcol nuclease
   - 64-74 µL TAG (use with PKRM) or TAG-M (use with RM) buffer
2. Vortex vigorously and incubate in a 24°C water bath for 15 minutes. Add 1 µL 100 mM EGTA to inhibit micrococcol nuclease.
3. Add 10 µL 10% TX-100 (in TAG or TAG-M - final concentration will be 1%) and 0.5 µL 100mM DTT (final concentration of 0.5 mM). Vortex vigorously and place on ice for 1 hour.
4. Spin at 17K*xg* in a tabletop centrifuge for 30 minutes at 4°C to generate a supernatant (17KgS), containing solubilized protein, and insoluble debris pellet (17KgP). Suspend the 17KgP (insoluble debris) in 50 µL hot TLB (this is the 'D' fraction).
5. Layer the 17KgS over a 100 µL 0.5M sucrose cushion (made in the respective buffer + 1% TX-100) Spin at 400K*g* (100K rpm) in a TLA 100 rotor at 4°C for 30 minutes. From the top of the tube collect 150 µL 300KgS ('Load' fraction), 50 µL sucrose cushion ('C' fraction) and TCA precipitate. Suspend both T and M fractions in 50 µL hot TLB. Suspend the 400KgP (ribosome pellet- 'P' fraction) in 50 µL hot TLB.
6. Run 15 µL per lane for Western analysis.

TAG-M buffer
20 mM Tris·HCl pH 7.4
500 mM amino caproic acid
5 mM $Mg(OAc)_2$
10% (v/v) glycerol

TAG Buffer
20 mM Tris·HCl pH 7.4
500 mM amino-caproic acid
10% (v/v) glycerol

0.5 M sucrose cushion
(in TAG or TAG-M buffer + 1% TX100)
For 1 mL – 0.17 g

2.4.3.12. Kodak Image Station

Western blots were developed directly on the Kodak Image Station (standard filter 'Open') using enhanced chemiluminescence (ECL) reagents. Using Kodak 1D Image

software, Western blot exposure data was collected with the following settings: 10-20 captures of 30-second exposures; x and y binning 'on'. Bands to be analyzed were boxed and Kodak 1D software was used to identify lanes and bands. Band profile width was adjusted manually and band sensitivity was set at zero. Kodak 1D software 'fit' the bands through the use of a Guassian distribution model. The net intensity of each band was determined using 1D software, and the data was analyzed using Microsoft Excel.

2.4.3.13. Assay for ribosome-PCC co-migration

Adapted from Meyer et al., 2000 (see Bibliography)

This assay uses sucrose gradient velocity centrifugation to analyze co-migration of translocon components

1. Add the following components to an eppendorf tube:
   20-40 µL RM (500- 1000 A_{280} µUnits)
   1 µL 200X PIN
   1 µL 100 mM PMSF
   10 µL 10% TX-100 in TAG-M buffer
   up to 100 µL with TAG-M buffer
2. Vortex vigorously and place on ice for 1 hour.
3. Spin for 5 minutes at 17K*xg* in a tabletop centrifuge at 4°C to generate a supernatant (17KgS), containing solubilized protein, and insoluble debris pellet (17KgP). The 17KgP can be discarded.
4. Layer 17KgS over a 2 mL 20%-40% continuous sucrose gradient made in a TLS 55 tube.
5. Spin at 55K rpm (~260K*xg*) for 1 hour at 4°C in a TLS 55 rotor.
6. Collect 100 µL fractions from the top of the gradient, TCA precipitate and suspend in 25 µL hot TLB. Run 15 µL per lane for Western analysis.

TAG-M buffer

20 mM Tris·HCl pH 7.4
500 mM amino-caproic acid
5 mM $Mg(OAc)_2$
10% (v/v) glycerol

40% sucrose in TAG-M + 1% TX-100

For 30 mL

0.3 mL	2 M Tris·HCl pH 7.4
0.75 mL	2 M amino-caproic acid
0.3 mL	0.5 M $Mg(OAc)_2$
6 mL	50% glycerol
3 mL	10% TX-100 in TAG-M buffer
12 g	sucrose

3. Results

3.1. Control of Sss1p expression in yeast

It has been previously described that properly targeted and functional Sss1p is necessary for yeast viability (Esnault et al., 1993). We have used this phenotype to develop a genetic assay in yeast to identify mutants of Sss1p that are defective in targeting to and/or function at the ER. The haploid yeast strain FKY M61 (see Table 1) has the single genomic copy of Sss1p knocked out and replaced by plasmid borne wild-type (Wt) Sss1p under the control of the *GAL10* promotor. The *GAL10* promotor is a repressor mediated system - in the absence of galactose the repressor binds to the promotor, blocking transcription; however when galactose is present it binds to the repressor, leading to dissociation from the promotor element, thus allowing transcription to occur.

Table 1. Yeast Strains

Strain	Genotype	Source
FKY 128	*sss1*::URA3/*SSS1* leu2-3,-112/leu2-3,-112 ade2-1/ade2-1 ura3-1/ura3-1 trp1-1/trp1-1 his3-11,-15/his3-11,-15 can1-100/can1-100 *MATα*/*MATa*	Esnault et al., (1993)
FKY 173	*sss1*::URA3 leu2-3,-112 ade2-1 ura3-1 his3-11,-15 trp1-1 can1-100 MATα p[*GAL10*::*SSS1*, *ADE2*]	Esnault et al., (1993)
FKY M61	same as FKY 173 p[*SEC61myc*, *TRP1*]	This study
FKY 198	*sss1*::*URA3* pep4::*LEU2* leu2-3,-112 ade2-1 ura3-1 his3-11,-15 trp1-1 can1-100 *MATα* p[*GAL10*::*SSS1*, *ADE2*] p[TPI::*SUC2*, *TRP1*]	Esnault et al., (1993)
RSY 763	*sec61*::HIS3 -1 trp1-1 leu2-3,112 ade2-1 his3-11,-15 p[GAL::*SEC61*]	Jeff Brodsky

As shown in Figure 6A, when FKY M61 were plated on galactose containing medium (YEPG) they grew normally, however when plated on medium containing glucose (YEPD) they did not grow. This phenotype on YEPD was rescued when yeast were complemented by a plasmid with the *SSS1* gene under the control of its endogenous promotor.

The signal sequence carrying proteins pre-Kar2p (pKar2p) and the mating pheromone pre-pro-alpha factor (ppαF), are targeted co- and post-translationally to the ER respectively (Ng et al., 1996). As pKar2p is transported into the ER lumen, the signal sequence is cleaved off to form the smaller mature Kar2p protein. PpαF is processed in the ER lumen to the mature, glycosylated alpha factor and is quickly secreted from the cell; it is therefore not detected in translocation competent cells by Western blotting. Whole cell lysates of yeast grown in YEPD medium were subjected to Western blot analysis (Figure 6B). After 4 hours a Kar2p doublet was observed, indicative of a block in pKar2p, and therefore co-translational transport. At the same time an accumulation of ppαF was observed, demonstrating a block in post-translational transport (Figure 6B – closed arrow). Sss1p expression was completely repressed after 6 hours (Figure 6B – open arrow). Furthermore, yeast depleted of Sss1p mounted an unfolded protein response, as shown by elevated expression of pKar2p (Tachibana & Stevens, 1992).

Figure 6

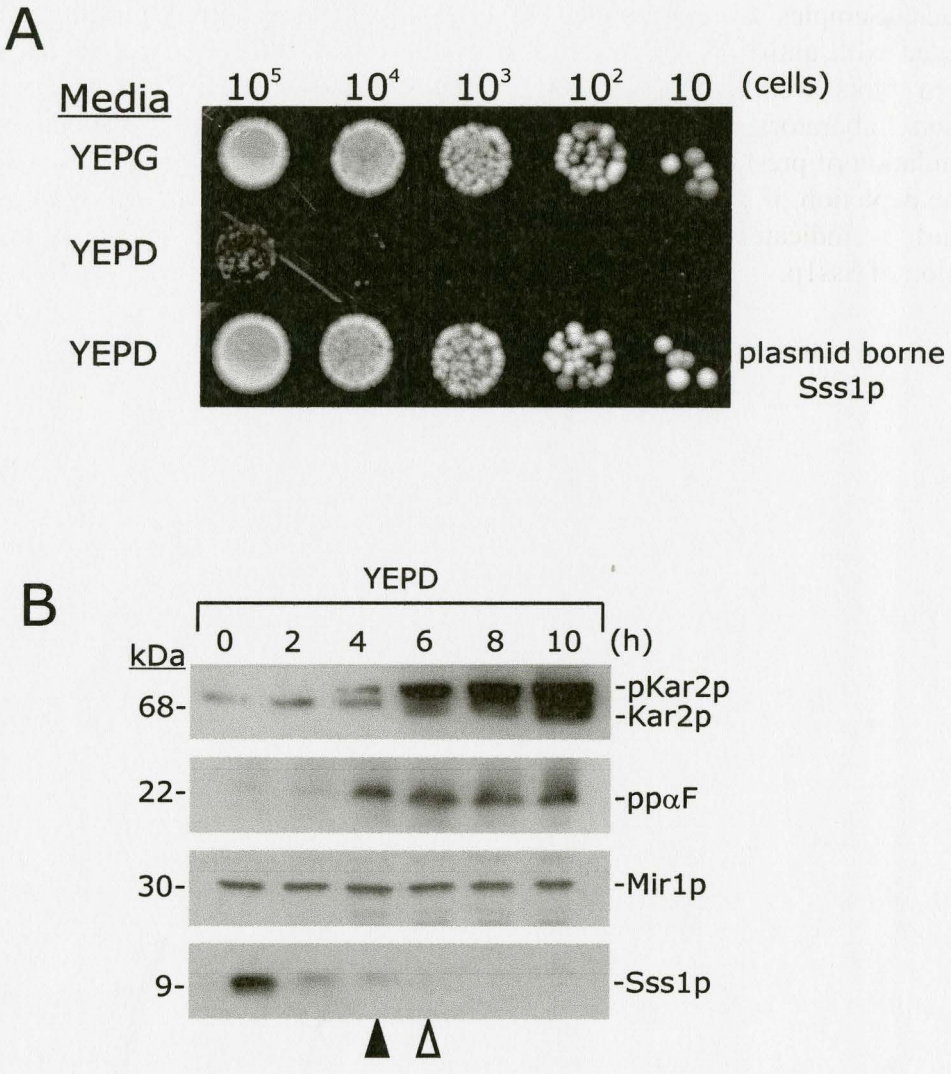

A
Media
YEPG
YEPD
YEPD
10^5
10^4
10^3
10^2
10
(cells)
plasmid borne
Sss1p
B
YEPD
0
2
4
6
8
10
(h)
kDa
68-
-pKar2p
-Kar2p
22-
-ppαF
30-
-Mir1p
9-
-Sss1p

Figure 6: Sss1p expression is essential for growth and can be controlled through the use of the repressible GAL10 promotor

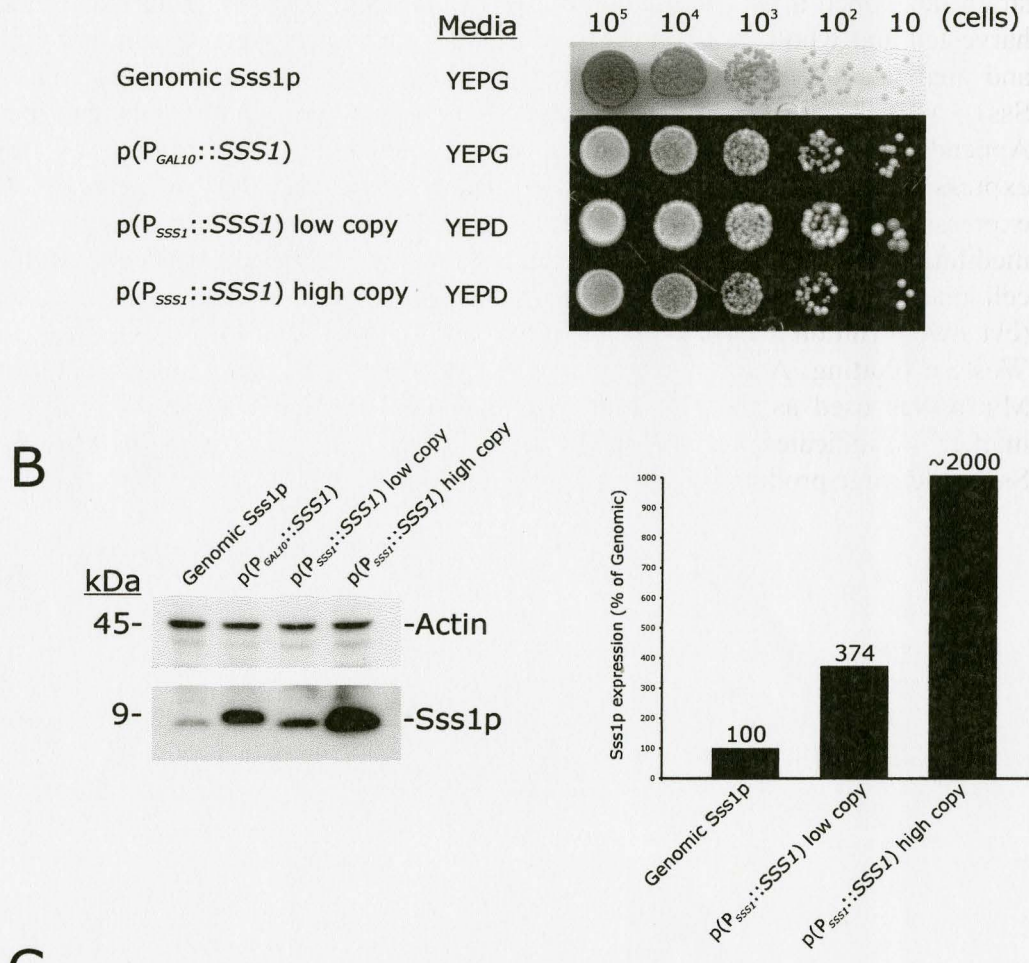
(A) *Yeast Growth Assay:* Yeast strain FKY M61, which has a single copy of *SSS1* under control of the *GAL10* promotor, was spotted on plates containing galactose (YEPG) or glucose (YEPD) medium and were grown at 30°C for 2 to 3 days. (B) *Western analysis of yeast whole cell lysates:* FKY M61 grown overnight in YEPG liquid medium at 30°C were switched to YEPD medium and grown for 0, 2, 4, 6, 8 and 10 hours at 30°C. Cells were harvested and whole cell lysates were prepared as described in Materials and Methods. Samples were separated by SDS-PAGE, transferred to nitrocellulose and decorated with anti-Kar2p, anti-ppαF, anti-Mir1p and anti-Sss1p polyclonal antibodies. Western blots were developed using donkey anti-rabbit HRP secondary antibodies (Jackson Laboratories) and enhanced chemiluminescence (ECL) reagents. The initial accumulation of pre-Kar2p (pKar2p) and pre-pro alpha factor (ppαF) (closed arrow head) and the depletion of Sss1p (open arrow head) are indicated. Mir1p was used as a loading standard. ▲ indicates beginning of the translocation defect. △ indicates the complete depletion of Sss1p.

We examined yeast expressing Wt Sss1p at different levels to determine if over-expression would have an effect on Sss1p targeting and/or function, which could subsequently influence yeast growth. The growth of yeast expressing Sss1p from the genome, from a plasmid under the *GAL10* promotor and from low and high-copy number plasmids under the endogenous *SSS1* promotor, did not differ (Figure 7A), even though Sss1p expression levels varied significantly (Figure 7B). To analyze the effects of expression levels on Sss1p targeting, we fractionated yeast into heavy membranes (HM), light membranes (LM) and cytosol (cyt. – the term cytosol refers to the cytoplasm extracted from the cell) (Figure 7C). Equal cell numbers of each fraction were loaded on a SDS-PAGE gel and were analyzed by Western blotting. As expected, Sss1p and Sec61p were predominantly found in the LM [primarily ER – also referred to as Rough Microsomes (RM)] of yeast expressing genomic levels of Sss1p. However as Sss1p expression was increased, greater amounts of Sss1p and Sec61p were found co-fractionating with the mitochondrial protein Mir1p in the HM fraction (Figure 7C – closed arrows). Over-expression of Sss1p did not lead to accumulation of the protein in the cytosol, as it did not co-fractionate with the cytosolic protein Actin. In addition, at expression levels 20-fold higher than genomic expression [p(P_{SSS1}::*SSS1*) high copy], an Sss1p cleavage product of 7 kDa was observed (Figure 7C - *). This degradation is a by-product of the fractionation process, as it is not observed in yeast whole cell lysates (Figure 7B). Therefore the expression of Sss1p above genomically expressed levels does not influence yeast growth, however it does influence the distribution of both Sss1p and Sec61p within the cell and it increases Sss1p's susceptibility to degradation. These effects were minor when Sss1p was expressed from its own promotor on a low-copy plasmid.

3.2. Yeast depleted of Sss1p can be rescued by re-introducing Sss1p

Using FKY M61 it was possible to test if yeast depleted of Sss1p could grow if Sss1p was re-introduced in the cell. Under conditions where endogenous Sss1p expression was repressed, FKY M61 carrying a plasmid with Sss1p under the control of its endogenous promotor (Wt) grew, while yeast which carried an empty plasmid (Vector) did not grow (Figure 8A – YEPD). When yeast were depleted of Sss1p after 8 hours of growth in YEPD liquid medium, an accumulation of pKar2p was observed by Western blotting (Figure 8B – YEPD), indicating a defect in translocon function. When these yeast were then switched from YEPD to YEPG liquid medium, endogenous Sss1p was expressed; after 15 hours no pKar2p was observed, thus demonstrating that translocon function had been restored (Figure 8B – Shift to YEPG). Sss1p depleted yeast also grew when switched to YEPG plates (Figure 8A – Shift to YEPG). Therefore yeast cells depleted of Sss1p for 8 hours are still alive, but will grow only if a functional Sss1p is re-introduced into the cell. Of interest is the observation that the re-introduced Sss1p was properly targeted and inserted into the ER membrane apparently independent of a functional translocon.

Figure 7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p(P_GAL10::SSS1)
YEPG
p(P_SSS1::SSS1) low copy
YEPD
p(P_SSS1::SSS1) high copy
YEPD
B
kDa
Genomic Sss1p
p(P_GAL10::SSS1)
p(P_SSS1::SSS1) low copy
p(P_SSS1::SSS1) high copy
45-
-Actin
9-
-Sss1p
Sss1p expression (% of Genomic)
100
374
~2000
Genomic Sss1p
p(P_SSS1::SSS1) low copy
p(P_SSS1::SSS1) high copy
C
Genomic Sss1p
p(P_SSS1::SSS1) low copy
p(P_SSS1::SSS1) high copy
HM
LM
cyt.
kDa
45-
30-
45-
9-
-Actin
-Mir1p
-Sec61p
-Sss1p
*

Figure 7: Over-expression of wild-type Sss1p does not reduce yeast growth

(A) Cells expressing Sss1p from the genome (Genomic Sss1p), and from low or high-copy number plasmids under control of the *GAL10* promotor [p(P_{GAL10}::*SSS1*)] or the endogenous *SSS1* promotor [p(P_{SSS1}::*SSS1*)] were spotted on YEPG or YEPD plates and grown at 30°C for 2 to 3 days. The FKY 128 image contrast differs from the others as this strain developed a pink pigmentation. (B) Cells from each Sss1p expression strain were harvested and whole cell lysates were prepared. Samples were separated by SDS-PAGE and analyzed by quantitative Western blotting using anti-Actin monoclonal, and anti-Sss1p polyclonal antibodies. Standard curves for these antibodies can be found in Appendix 1. The amount of Sss1p was normalized to the amount of Actin. Sss1p expression from each strain was presented graphically as a percent of genomic expression, with genomic expression set to 100%. (B) (C) Yeast grown at 30°C in YEPD medium for 12 hours were fractionated as described in the Materials and Methods. Equal cell numbers of purified heavy membranes (HM), light membranes (LM) and cytosol (cyt.) were run on a 10% SDS-PAGE gel and the localization of Sss1p was analyzed by Western blotting. Actin was used as the cytoplasmic marker, the mitochondrial protein Mir1p was used as the HM marker and the ER protein Sec61p was used as the LM marker. ▲ indicates the mislocalization of Sec61p and Sss1p to the HM fraction. An Sss1p cleavage product is indicated by '*'.

Figure 8

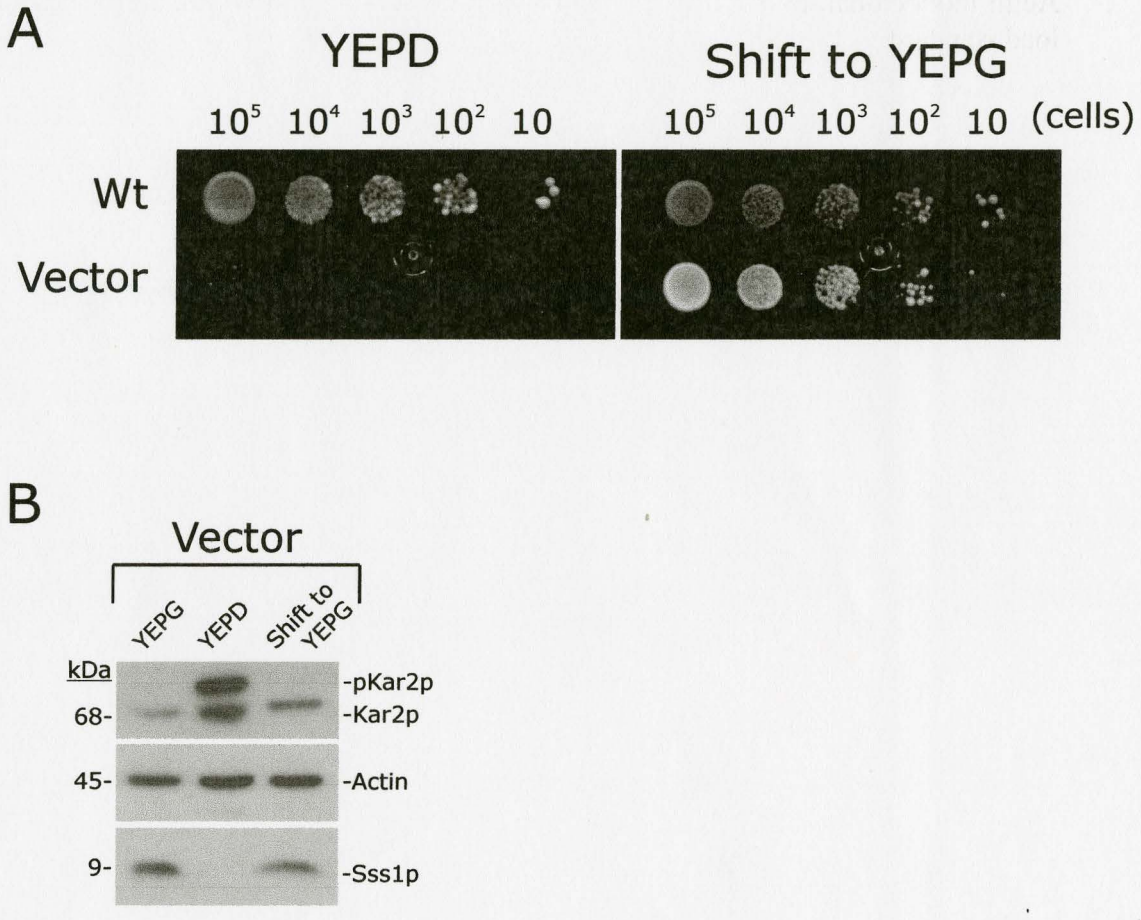

A
YEPD
Shift to YEPG
10^5 10^4 10^3 10^2 10
10^5 10^4 10^3 10^2 10 (cells)
Wt
Vector
B
Vector
YEPG
YEPD
Shift to YEPG
kDa
68-
45-
9-
-pKar2p
-Kar2p
-Actin
-Sss1p

Figure 8: Yeast depleted of Sss1p can be rescued by re-introducing Sss1p

(A) Yeast strain FKY M61 carrying a plasmid with *SSS1* expressed under the endogenous *SSS1* promotor (Wt) or an empty plasmid (Vector) were spotted on plates and grown at 30°C for 2 to 3 days (YEPD). FKY M61 initially grown for 8 hours in YEPD medium at 30°C (to deplete endogenous Sss1p), were also spotted on plates and grown at 30°C for 2 to 3 days (Shift to YEPG). (B) FKY M61 carrying an empty plasmid (Vector) were grown at 30°C for 8 hours in YEPG, YEPD, or for 8 hours in YEPD and then 15 hours in YEPG (Shift to YEPG). Cells were harvested and whole cell lysates were prepared. Samples were run on 10% SDS-PAGE gels and analyzed by Western blotting using anti-Actin monoclonal, anti-Kar2p and anti-Sss1p polyclonal antibodies. Actin was used as a load standard.

3.3. Depletion of essential PCC components leads to loss of ribosome binding to the ER and accumulation of translocon proteins in heavy membranes

We investigated how the depletion of the essential PCC components Sec61p and Sss1p affected the localization of translocon proteins in the cell. Yeast expressing a single copy of either *SEC61* or *SSS1* on a plasmid under control of the galactose promotor did not grow on YEPD medium (Figure 9A – compare Wt to pGAL::*SEC61* and pGAL::*SSS1* respectively). Yeast depleted of Sec61p displayed a similar defect in both co- and post-translational transport as yeast depleted of Sss1p (Figure 9B). After 20 hours in YEPD medium, Western blotting showed that Sec61p expression was completely repressed, and an accumulation of pKar2p and ppαF was observed (Figure 9B – closed circle) – indicative of a shutdown in co- and post-translational translocation. As previously reported, we observed that the depletion of Sec61p also had a destabilizing effect on Sss1p (Figure 9B – closed circle) (Esnault et al., 1994; Biederer et al., 1996).

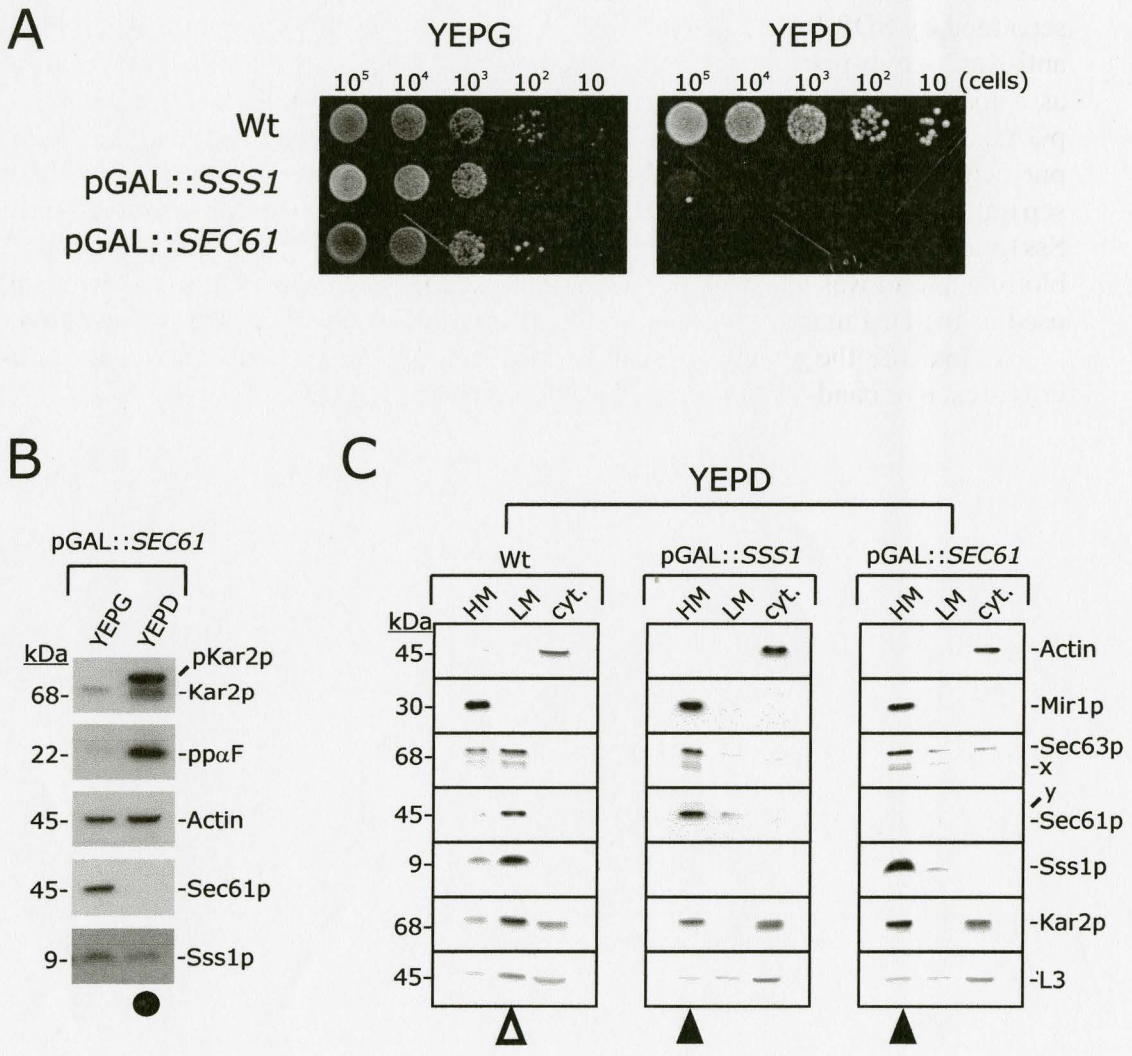
Yeast expressing Sss1p (Wt), and yeast depleted of Sss1p (pGAL::*SSS1*) or Sec61p (pGAL::*SEC61*) were fractionated into HM, LM and cytosol fractions. Each fraction, normalized to cell number, was loaded on a SDS-PAGE gel and subjected to Western blot analysis (Figure 9C). In Wt yeast, the translocon components Sec63p, Sec61p, Sss1p and the lumenal protein Kar2p were found predominantly in the LM fraction (open arrow), with some contaminating protein co-fractionating with the mitochondrial protein Mir1p in HMs. Some Kar2p was also found in the cytosolic fraction, however this was likely released by the disruption of the ER during the fractionation process. The large ribosomal sub-unit L3 was found in the LM and cytosol fractions, representative of the two known ribosome pools in the cell – cytoplasmic ribosomes and ER bound ribosomes.

Depletion of either Sss1p or Sec61p led to an accumulation of ER proteins in the HM fraction (Figure 9C – closed arrows), and the accumulation of pKar2p in the cytosol. A surprising observation was the accumulation of pKar2p in the HM fraction as well, even though in translocation defective cells precursor proteins accumulate in the cytosol. There was also a significant decrease in ribosomes associated with the LM fraction. Ribosomes were instead found predominantly in the cytosol, with some also localized to the HM fraction. Therefore the depletion of either essential PCC component led to the mislocalization of translocon components to the HM fraction and caused a defect in ribosome association with the ER.

3.4. Depletion of Sss1p decreases ribosome binding to Sec61p

To further investigate the defect in ribosome binding to the ER, membranes were purified from Wt and Sss1p-depleted yeast. Since Sec61p is the main ribosome receptor on the ER, and Sss1p is known to interact with the region of Sec61p that may be involved in ribosome binding (this region has been shown to be involved in ribosome binding in mammals), we analyzed the importance of Sss1p in ribosome binding to Sec61p. We first assayed the ability of Sec61p to pellet with ribosomes. 250 or 500 A_{280} μUnits (μU) of RMs or puromycin/high-salt treated rough microsomes (PKRM) were solubilized in 1% TX-100, layered over a 0.5 M sucrose cushion and subjected to centrifugation at

Figure 9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YEPD
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10⁵ 10⁴ 10³ 10² 10 (cells)
Wt
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pGAL::SEC61
B
pGAL::SEC61
YEPG
YEPD
kDa
pKar2p
68-
-Kar2p
22-
-ppαF
45-
-Actin
45-
-Sec61p
9-
-Sss1p
C
YEPD
Wt
pGAL::SSS1
pGAL::SEC61
HM
LM
cyt.
kDa
45-
30-
68-
45-
9-
68-
45-
-Actin
-Mir1p
-Sec63p
-x
-y
-Sec61p
-Sss1p
-Kar2p
-L3

Figure 9: Yeast depleted of essential PCC components show mislocalization of translocon proteins and are defective in ribosome binding to the ER

(A) Yeast strain FKY M61 carrying a plasmid with *SSS1* expressed under the endogenous *SSS1* promotor (Wt) or under the galactose promotor (pGAL::*SSS1*), and strain RSY 763 carrying a plasmid with *SEC61* expressed under the galactose promotor (pGAL::*SEC61*) were spotted on plates containing either galactose (YEPG) or glucose (YEPD) and grown at 30°C for 2 to 3 days. (B) RSY 763 was grown for 20 hours in YEPG or YEPD liquid medium. Cells were harvested and whole cell lysates were prepared. Samples were separated by SDS-PAGE and analyzed by Western blotting using anti-Actin monoclonal, anti-Kar2p, anti-ppαF, anti-Sec61p and anti-Sss1p polyclonal antibodies. Actin was used as a load standard. (C) Yeast grown at 30°C in YEPD medium for 12 hours (Wt and pGAL::*SSS1*) or 20 hours (pGAL::*SEC61*) were fractionated. Equal cell numbers of purified heavy membranes (HM), light membranes (LM) and cytosol (cyt.) were separated by SDS-PAGE and the localization of the translocon proteins Sec63p, Sec61p, Sss1p and Kar2p, as well as the large ribosomal subunit L3, was determined by Western blotting. Actin was used as the cytosolic marker and the mitochondrial protein Mir1p was used as the HM marker. ● indicates the translocation defect caused by Sec61p depletion. △, ▲, indicate the principal location of translocon components. 'X' is an anti-Sec63p cross-reactive band. 'Y' is an anti-Sec61p cross-reactive band.

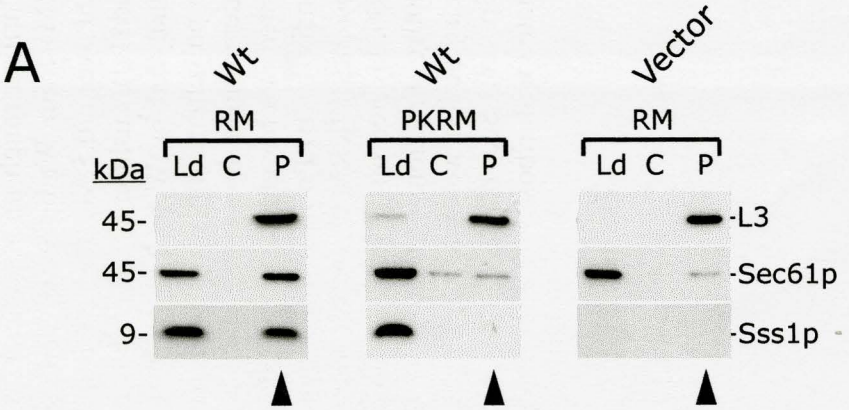
400,000*xg*. Load (Ld), sucrose cushion (C) and ribosome pellet (P) fractions were prepared and analyzed by Western blotting. The percent of total Sec61p in the ribosome pellet was determined by quantitative Western analysis and presented graphically in Figure 10A. Appendix 1 includes the standard curve of the anti-Sec61p polyclonal antibodies used for quantitative Western analysis. In Wt RM 61% of total Sec61p co-fractionated with the large ribosomal subunit L3, while in Wt PKRM, where ribosomes have been stripped from the PCC, only 8% of Sec61p associated with L3. In Sss1p depleted RMs (Vector), ribosome associated Sec61p dropped by a similar amount, to 12% of the total (Figure 10A – closed arrows).

The co-migration of ribosomes with the PCC can also be examined using a sucrose gradient (Meyer et al., 2000). To further characterize the observed disruption of the ribosome-Sec61p complex, we next examined the effect of Sss1p depletion on this co-migration pattern. 500 or 1000 A_{280} μU of RMs or PKRMs were solubilized in 1% TX-100, layered on top of a continuous 20% to 40% sucrose gradient and subjected to velocity centrifugation at 230,000*xg*. Fractions were collected from the top of the gradient and analyzed by Western blotting (Figure 10B). Both Sec61p and Sss1p co-migrated with the large ribosomal subunit L3 when solubilized Wt RM were assayed (lanes 8–9, closed arrows). The ER membrane protein Sec63p does not associate with ribosomes (Meyer et al., 2000) and therefore remained in the top fractions (lanes 1-2) as expected. When ribosomes were stripped off of the PCC (Wt PKRM), Sec61p and Sss1p no longer co-migrated with L3 but remained at the top of the gradient with Sec63p (compare lanes 8-9, closed arrows, with lanes 1-2). Depletion of Sss1p (Vector) prevented Sec61p co-migration with L3 (compare lanes 8-9, closed arrows, with lanes 1-2). These results demonstrate that Sss1p plays an essential role in ribosome binding to Sec61p. It remains to be determined if depletion of Sss1p effects the integrity of the PCC (see Discussion). Nevertheless, these assay-systems can be used to examine the effect of mutations in the TA of Sss1p.

3.5. Sss1p carries a targeting signal similar to VAMP-1A

Figure 11A presents a schematic representation of the Sss1p mutant proteins used in this study. Figure 11B presents a table that summarizes the results found in Figures 12 through 18. Kim et al., 1999, have demonstrated that the ER targeting signal for VAMP-1A is located in the NTS, and is composed of four lysine residues that form an amphipathic helix. The NTS of Sss1p is also predicted to form an amphipathic helix (Beswick et al., 1998) that resembles the VAMP-1A targeting signal, with 5 lysine residues facing one side of the helix (Figure 12A - box). To determine if the lysine residues comprise the ER targeting signal for Sss1p, all five lysines were mutated to threonine residues. As shown in Figure 12B, this mutant displayed the same growth phenotype as Wt Sss1p (compare Sss1pK_5/T_5 with Wt), even though it was expressed at less than half the level of Wt (Figure 12C). Fractionation of this mutant showed that it was predominantly targeted to the HM fraction without the redistribution of Sec61p observed in Sss1p-depleted yeast (Figure 12D – closed arrow). Therefore enough of the Sss1pK_5/T_5 mutant protein was targeted to the ER to allow for Wt growth. We conclude that Sss1p has an ER targeting signal similar to VAMP-1A.

Figure 10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Wt
RM
Wt
PKRM
Vector
RM
kDa
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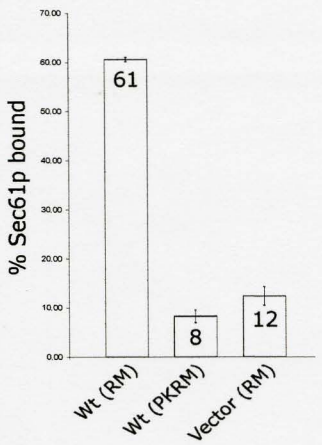
Ld C P
Ld C P
45-
45-
9-
-L3
-Sec61p
-Sss1p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60.00
50.00
40.00
30.00
20.00
10.00
0.00
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12
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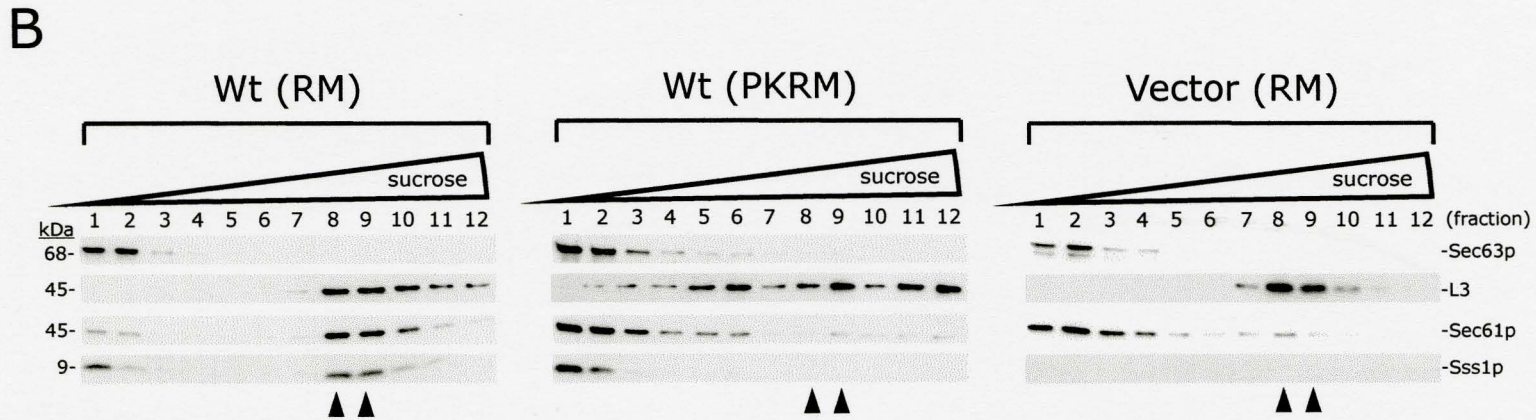
Wt (PKRM)
Vector (RM)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Wt (PKRM)
Vector (RM)
sucrose
kDa
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(fraction)
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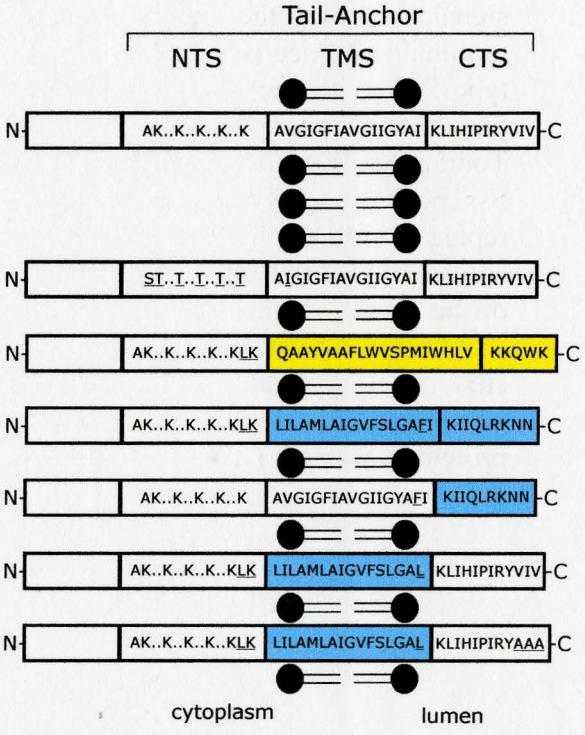
45-
45-
9-
-Sec63p
-L3
-Sec61p
-Sss1p

Figure 10: Depletion of Sss1p reduces efficient ribosome binding to Sec61p
(A) Purified rough microsomes (RM) or puromycin/high salt treated rough microsomes (PKRM) from Wt or Sss1p depleted yeast (Vector) were solubilized in 1% TX-100, layered over a 0.5 M sucrose cushion and centrifuged at 400,000*xg*. Equivalent volumes of load (Ld), cushion (C) and ribosome pellet (P) fractions were separated by SDS-PAGE and analyzed by Western blotting using anti-L3 monoclonal and anti-Sec61p and anti-Sss1p polyclonal antibodies. The percent of total Sec61p in the ribosome pellet fraction was determined by quantitative Western analysis using a Kodak Image Station and presented in a bar graph. The standard curve for the anti-Sec61p antibody can be found in Appendix 1. (B) Purified rough microsomes (RM) or puromycin/high salt treated rough microsomes (PKRM) from Wt or Sss1p depleted yeast (Vector) were solubilized in 1% TX-100, layered on top of a continuous 20% to 40% sucrose gradient and centrifuged at 230,000*xg*. Fractions were collected from the top of the gradient and equivalent volumes of each fraction were analyzed by Western blotting using anti-L3 monoclonal and anti-Sec63p, anti-Sec61p and anti-Sss1p polyclonal antibodies. ▲ indicates the proteins associated with ribosomes. ▲▲ indicates the location of Wt ribosome-PCC complexes.

Figure 11

A

Plasmid	Name
pMAC1150	Wt
pMAC1148	Vector
pMAC1537	Sss1pK_5/T_5
pMAC1395	Sss1pTom5
pMAC1401	Sss1pActA
pMAC1402	Sss1pCTSa
pMAC1459	Sss1pTMSa
pMAC1503	Sss1pCTSwtVIV/AAA



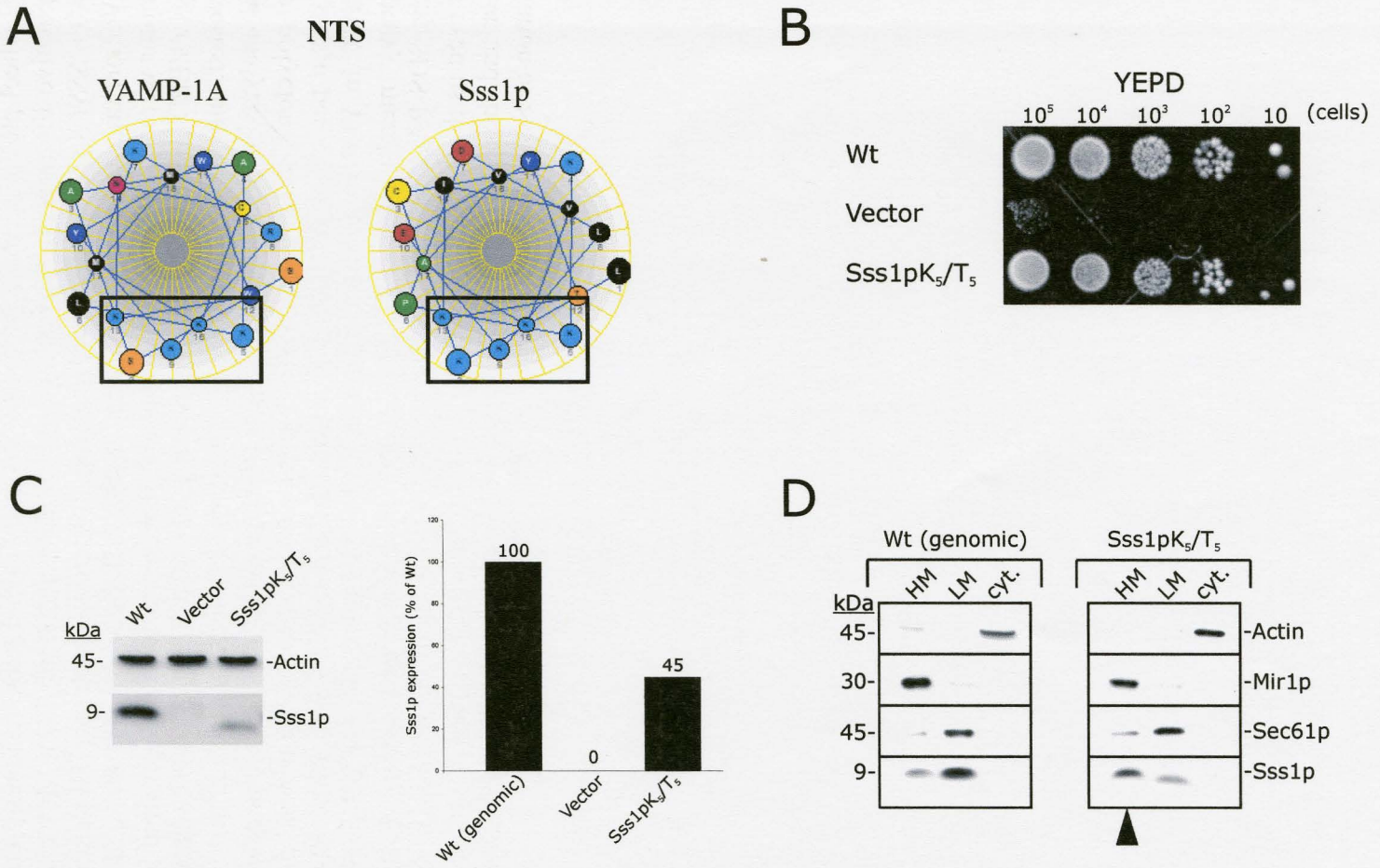
B

Plasmid	Growth	Localization		Bound to Ribosomes	
		Sec61p	Sss1p	% Sec61p	% Sss1p
pMAC1150	+++++	ER	ER	61 +/- 1	32 +/- 3
pMAC1148	–	Mito.	–	12 +/- 2	–
pMAC1537	+++++	ER	ER/Mito.	n/a	n/a
pMAC1395	+	Mito.	Mito.	n/a	n/a
pMAC1401	++	Mito.	Mito.	n/a	n/a
pMAC1402	++	Mito.	Mito.	29 +/- 4	0
pMAC1459	++++	ER/Mito.	ER/Mito.	19 +/- 3	0
pMAC1503	+	Mito.	Mito.	23 +/- 1	0

Figure 11: Sss1p mutants used in this study

(A) Schematic diagram of coding regions of Sss1p mutants used in this study. Sss1p is a tail-anchor (TA) protein with a TA located at the extreme carboxyl-terminus. The TA includes the transmembrane sequence (TMS) and the potential locations of the targeting signal, including the amino-terminal sequence (NTS), the TMS itself, or the carboxyl-terminal sequence (CTS). Mutations were engineered in the TA as follows: Wt – Wild-type Sss1p; Vector – no Sss1p; Sss1pK_5T_5 – 5 lysine residues in the NTS mutated to threonine residues; Sss1pTom5 – TMS and CTS of Sss1p replaced with TMS and CTS of Tom5p; Sss1pActA – TMS and CTS of Sss1p replaced with the TMS and CTS of ActA; Sss1pCTSa – CTS of Sss1p replaced with the CTS of ActA; Sss1pTMSa – TMS of Sss1p replaced with the TMS of ActA; Sss1pCTSwtVIV/AAA – Sss1pTMSa with the last 3 amino acids of the Wt CTS (V-I-V) mutated to alanine residues. The amino acid sequence of the TAs are indicated. Point mutations introduced to facilitate cloning are underlined. (B) Table summarizing the experimental results obtained for each Sss1p mutant in this study. [ER = predominantly endoplasmic reticulum (light membrane fraction); Mito. = predominantly mitochondria (heavy membrane fraction); '–' = no yeast growth or no protein expressed; n/a = not applicable].

Figure 12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NTS
VAMP-1A
Sss1p
B
YEPD
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Wt
Vector
Sss1pK5/T5
C
kDa
Wt
Vector
Sss1pK5/T5
45-
-Actin
9-
-Sss1p
Sss1p expression (% of Wt)
100
0
45
Wt (genomic)
Vector
Sss1pK5/T5
D
Wt (genomic)
Sss1pK5/T5
HM
LM
cyt.
kDa
45-
30-
45-
9-
-Actin
-Mir1p
-Sec61p
-Sss1p

Figure 12: Sss1p contains a targeting signal resembling VAMP-1A

(A) Helical wheel plot of the NTS of VAMP-1A and Sss1p. Lysine residues predicted to form the putative ER targeting signal are boxed. (B) Yeast strain FKY M61 carrying a plasmid with *SSS1* expressed under the endogenous *SSS1* promotor (Wt), an empty plasmid (Vector) and a plasmid carrying the Sss1pK_5/T_5 mutant under the endogenous *SSS1* promotor were spotted on plates containing glucose medium and grown at 30°C for 2 to 3 days (YEPD). (C) The same yeast were grown in YEPD medium for 8 hours at 30°C, harvested, and whole cell lysates were prepared. Samples were separated by SDS-PAGE and analyzed by quantitative Western blotting using anti-Actin monoclonal and anti-Sss1p polyclonal antibodies. Sss1p expression from each strain was presented graphically as a percent of Wt expression, with Wt expression set to 100%. (D) Yeast grown at 30°C in YEPD medium for 12 hours were fractionated. Equal cell numbers of purified heavy membranes (HM), light membranes (LM) and cytosol (cyt.) were separated by SDS-PAGE and the localization of Sss1p was analyzed by Western blotting. Actin was used as the cytosolic marker, the mitochondrial protein Mir1p was used as the HM marker and the ER protein Sec61p was used as the LM marker. ▲ indicates the mislocalization of Sss1pK_5T_5 to the HM fraction.

When the positively charged lysine residues in this targeting signal are replaced with threonine residues, targeting is dramatically reduced but not abolished. Even though these point mutations in the NTS of Sss1p do not affect its function, the complete replacement of the NTS with that from Tom22p was not tolerated as this mutant could not be stably expressed in yeast (Mark Campbell – Andrews' Lab, personal communication). Our results also demonstrate that remarkably little Sss1p needs to be targeted to the ER in order for yeast to grow normally.

3.6. Mutations in the TMS and CTS of Sss1p affect yeast growth

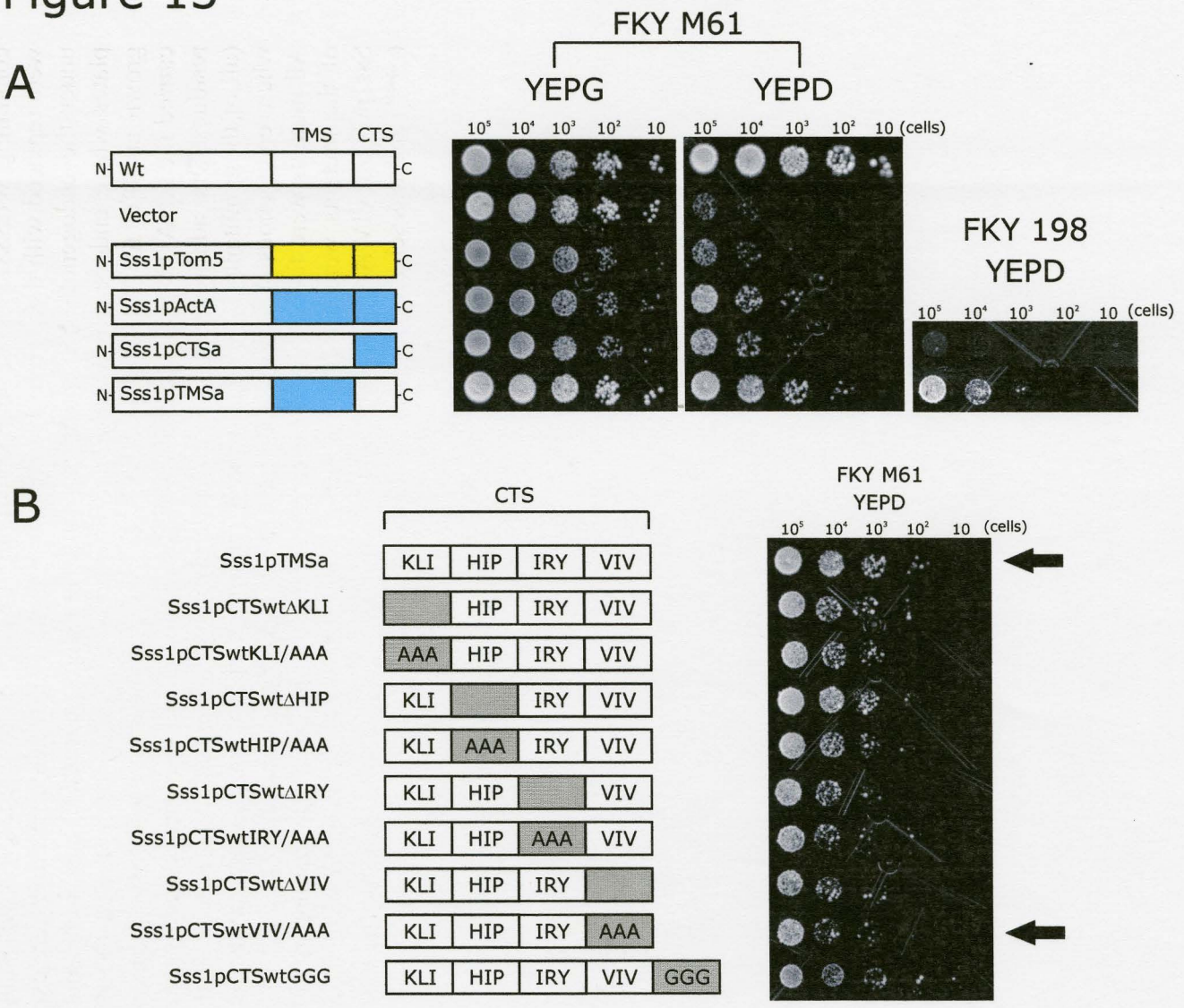
To further analyze the TA of Sss1p, we mutated the TMS and CTS to determine if they are involved in targeting or function. Sss1p mutants that substituted the TMS and/or CTS with those from ActA and Tom5p (see Figure 11A) were expressed in FKY M61 grown under conditions where endogenous Wt Sss1p was expressed or repressed (Figure 13A – YEPG and YEPD respectively). The TMS and CTS of ActA, a protein from the bacteria Listeria monocytogenes, have been shown together to be sufficient to target carrier proteins to the mitochondria in mammalian cells (Zhu, et al., 1996). The CTS of Tom5p, a TA protein that is a member of the yeast outer mitochondrial membrane translocase, was found to be sufficient to direct GFP to the mitochondria in mammalian cells as long as it was immediately preceded by a TMS (Horie et al., 2002).

All the mutants analyzed were recessive, in that yeast grew normally on YEPG medium. Expression of the Sss1pTom5 mutant protein resulted in a 10,000-fold decrease in yeast growth on YEPD medium. Both the Sss1pActA and the Sss1pCTSa mutant proteins caused a 1,000-fold defect in yeast growth compared to Wt, while expression of the Sss1pTMSa mutant protein, which contains the ActA TMS and Wt CTS, only resulted in approximately a 10-fold growth defect (Figure 11B). These results suggest that the CTS of Sss1p is essential for targeting and/or function of Sss1p at the ER. In contrast, the precise sequence of the TMS is less important for targeting or function. As will be described below, there was a surprising lack of correlation between yeast growth and ribosome binding (ex. compare pMAC1459 and pMAC1402 in Figure 11B).

To confirm the importance of the Wt Sss1p CTS for yeast growth, the Sss1pCTSa and Sss1pTMSa mutants were expressed in the yeast strain FKY 198 (see Table 1). This strain constitutively over-expresses the secretory protein Suc2p; it would therefore be expected that any defect in the transport of secretory proteins in this strain would lead to a rapid accumulation of pre-proteins in the cytoplasm, thereby increasing the rate of cell death. Only yeast that expressed the Sss1p mutant carrying the Wt CTS grew in this strain. As shown in Figure 13A, the growth of FKY 198 cells expressing Sss1pTMSa were further compromised than FKY M61 cells expressing the same mutant Sss1p protein.

We employed scanning mutagenesis to determine the importance of specific amino acids in the CTS of Sss1p (Figure 13B). We engineered Sss1p mutants that carried the ActA TMS and had groupings of three CTS amino acids either added, deleted or replaced with alanine or glycine residues. These mutants were expressed in the yeast strain FKY M61 and their growth analyzed. All the mutations, with the exception of Sss1pCTSwtGGG mutant, caused a decrease in yeast growth compared to the parent

Figure 13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FKY M61
YEPG
YEPD
TMS
CTS
N
C
Wt
Vector
Sss1pTom5
Sss1pActA
Sss1pCTSa
Sss1pTMSa
10⁵ 10⁴ 10³ 10² 10 (cells)
FKY 198
YEPD
B
CTS
Sss1pTMSa
Sss1pCTSwtΔKLI
Sss1pCTSwtKLI/AAA
Sss1pCTSwtΔHIP
Sss1pCTSwtHIP/AAA
Sss1pCTSwtΔIRY
Sss1pCTSwtIRY/AAA
Sss1pCTSwtΔVIV
Sss1pCTSwtVIV/AAA
Sss1pCTSwtGGG
KLI
HIP
IRY
VIV
AAA
GGG
FKY M61
YEPD

Figure 13: Mutations in the TMS and CTS of Sss1p inhibit yeast growth

(A) Yeast strains FKY M61 and FKY 198 were transformed with either a plasmid containing Wt *SSS1* or a plasmid containing the *SSS1* gene where the TMS and/or CTS were replaced with those from Tom5p or ActA. All *SSS1* mutant proteins were expressed under the endogenous *SSS1* promotor on a low-copy plasmid. Yeast were spotted on plates where endogenous Wt Sss1p was expressed (YEPG) or repressed (YEPD) and grown at 30°C for 2 to 3 days. (B) Yeast were transformed with Sss1p mutants that carried the ActA TMS and had amino acids in the CTS deleted or replaced with alanine residues. One additional mutant had three glycine residues added to the end of the CTS (all of these mutants were engineered by Paulina Dlugosz). Yeast were spotted on plates where endogenous Wt Sss1p was repressed (YEPD) and grown at 30°C for 2 to 3 days. All mutants were found to be recessive. Western blot analysis also demonstrated that all of the mutants were stably expressed (data not shown). Yeast expressing the mutant Sss1pCTSwtVIV/AAA showed 100-fold lower growth than the parent Sss1pTMSa strain (⬅) and was selected for further study.

mutant Sss1pTMSa. Replacing the last three amino acids with alanine residues caused the most dramatic growth defect (Sss1pCTSwtVIV/AAA), with an approximate 1000-fold decrease in yeast growth compared to the parent mutant. Since this mutant protein maintains the Wt length of the CTS (12 amino acids), it was selected for further analysis. This mutant was used to determine whether the last three amino acids of the CTS are important for Sss1p stability, targeting or function.

3.7. Sss1p mutants are stable and expressed in cells at higher levels than Wt

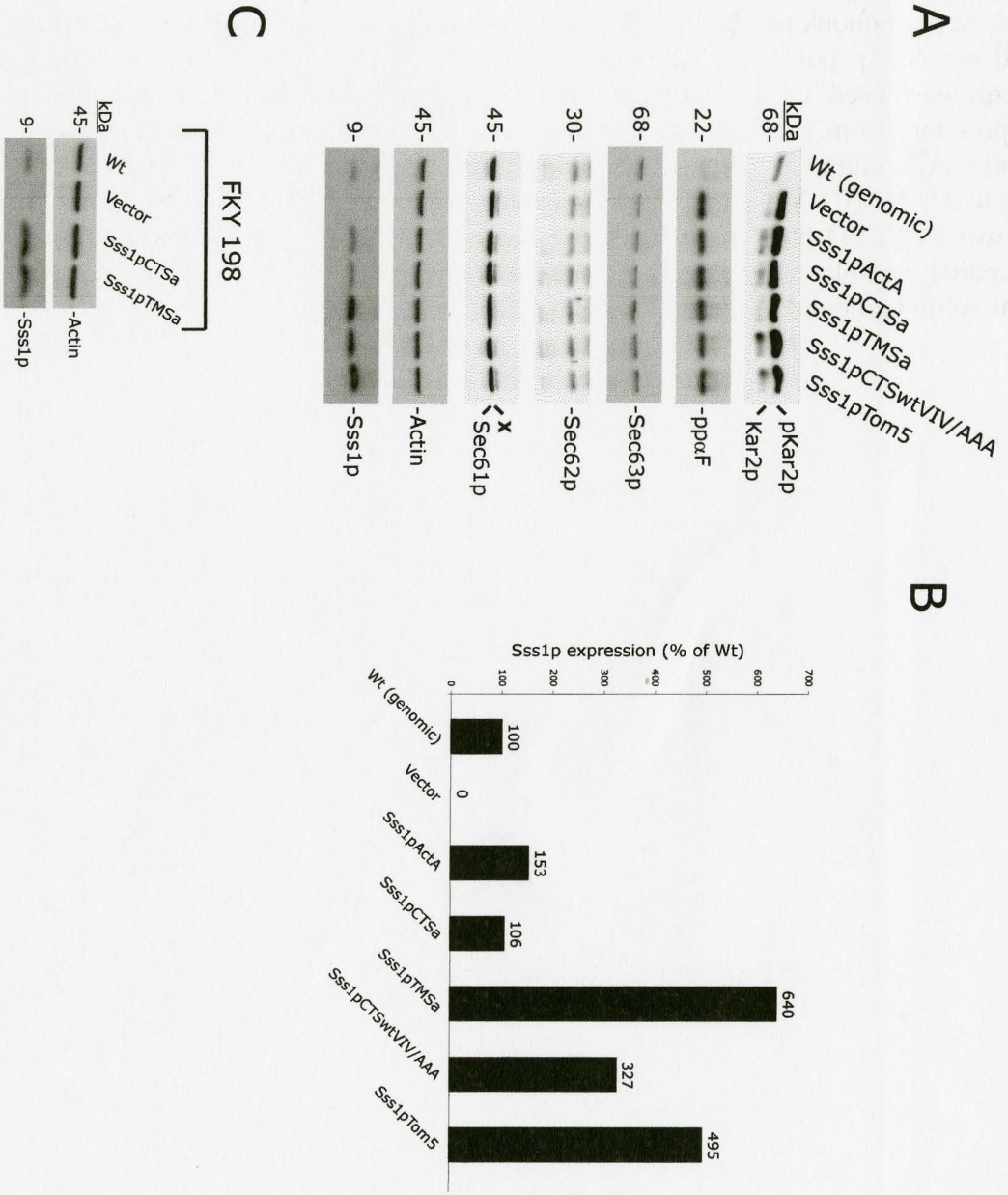
To determine if the decrease in growth observed with yeast expressing mutant Sss1p proteins correlated with the expression levels in cells, whole cell lysates were prepared and subjected to Western blot analysis. All Sss1p mutants were expressed at higher levels than Wt (Figure 14A and 14C), and the expression levels determined by quantitative Western blotting did not correlate with growth (Figure 14B). Appendix 1 includes the standard curve of the anti-Sss1p polyclonal antibodies used for quantitative Western analysis. All mutants also demonstrated a build-up of pKar2p and ppαF, indicating a defect in co- and post-translational transport (Figure 14A). The expression levels of the essential translocon proteins Sec61p, Sec62p and Sec63p did not significantly differ in yeast expressing mutant Sss1p compared to Wt yeast, however there was a reproducible anti-Sec61p cross-reactive band (Figure 14A – Band 'X') that was only found in yeast depleted of Sss1p or expressing Sss1p mutant protein.

3.8. Yeast expressing Sss1p mutants lacking the Wt CTS show mislocalization of translocon proteins to HMs

We fractionated yeast expressing Sss1p mutants to determine if mistargeting of Sss1p away from the ER caused the observed decrease in growth (Figure 15). Yeast expressing the Sss1pTom5, Sss1pActA, Sss1pCTSa and Sss1pCTSwtVIV/AAA mutants all showed similar fractionation patterns as Sss1p depleted yeast (Vector), with translocon proteins predominantly co-fractionating with the mitochondrial protein Mir1p (closed arrow). All of these mutant Sss1p proteins were susceptible to proteolytic degradation (*), while Wt Sss1p was not degraded (open arrow). In addition, these mutants showed a decrease in ribosomes in the LM fraction. Instead, ribosomes were found in the cytosol and HM fractions. This result was surprising as ribosomes were not detected in the HM fraction in Wt or Sss1p depleted cells. Yeast expressing the Sss1pTMSa mutant showed a slightly different fractionation pattern. Although these yeast had mislocalized translocon components to the HM fraction, a significant amount of Sec61p and Sss1p, as well as Sec63p, were properly localized to the LM fraction (compare closed circles). The Sss1pTMSa mutant proteins were also susceptible to proteolytic cleavage (*). Unlike yeast expressing other Sss1p mutant proteins, ribosomes were not found in the HM fraction of the Sss1pTMSa mutant.

The results suggest a correlation between yeast growth and the mislocalization of translocon components. The Sss1pTom5, Sss1pActA, Sss1pCTSa and Sss1pCTSwtVIV/AAA expressing yeast had severe growth defects and mislocalized most of the ER proteins analyzed to the HM fraction. The Sss1pTMSa expressing yeast showed

Figure 14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kDa
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22-
68-
30-
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9-
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Vector
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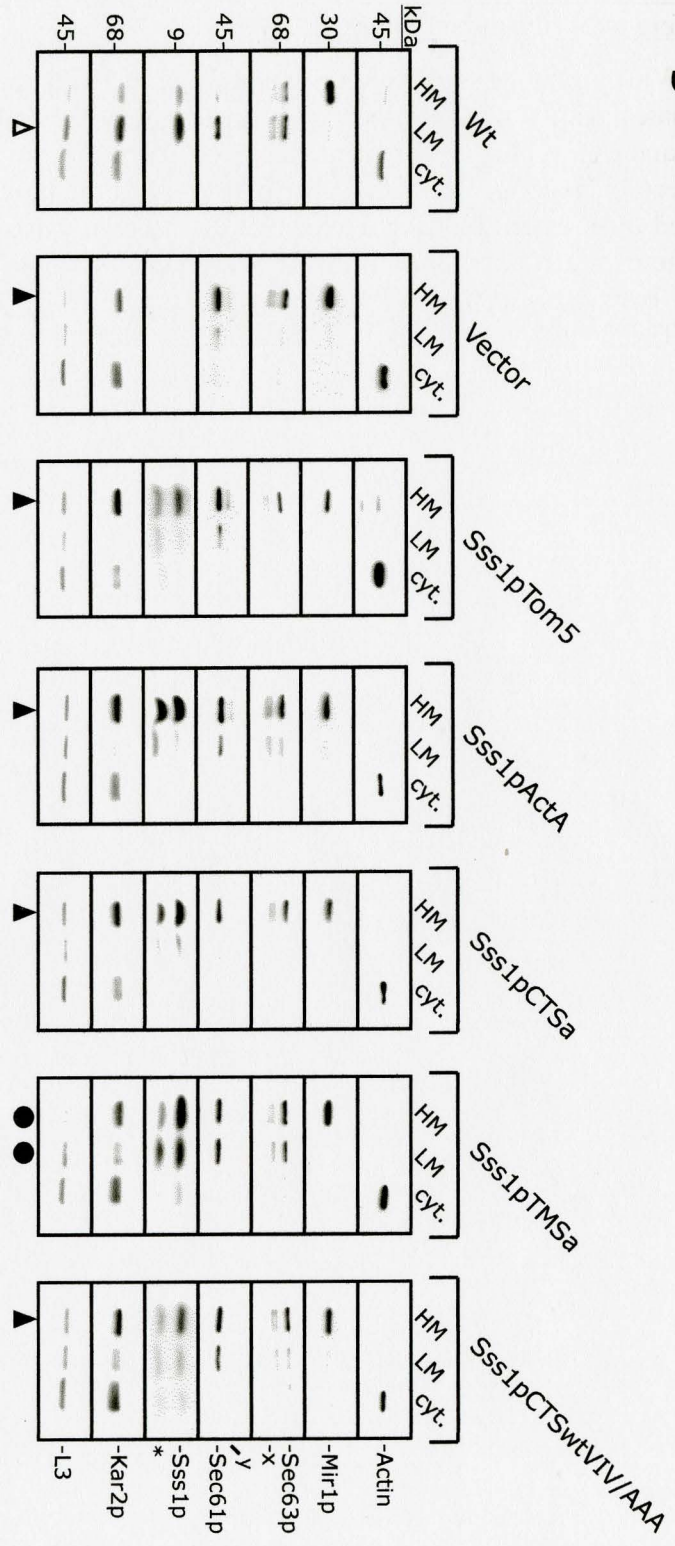
pKar2p
Kar2p
-ppαF
-Sec63p
-Sec62p
Sec61p
-Actin
-Sss1p
B
Sss1p expression (% of Wt)
0
100
200
300
400
500
600
700
Wt (genomic)
Vector
Sss1pActA
Sss1pCTSa
Sss1pTMSa
Sss1pCTSwtVIV/AAA
Sss1pTom5
100
0
153
106
640
327
495
C
FKY 198
kDa
45-
9-
Wt
Vector
Sss1pCTSa
Sss1pTMSa
-Actin
-Sss1p

Figure 14: Sss1p mutants are stable and expressed at higher levels than Wt Sss1p

(A) Yeast strain FKY 128 (Wt – genomic) and strain FKY M61 expressing Sss1p mutants were grown in YEPD medium for 8 hours at 30°C, harvested, and whole cell lysates were prepared. Samples were separated by SDS-PAGE and analyzed by Western blotting using anti-Actin monoclonal and anti-Kar2p, anti-ppαF, anti-Sec63p, anit-Sec62p, anti-Sec61p and anti-Sss1p polyclonal antibodies. Band 'X' is an anti-Sec61p cross-reactive band. Actin was used as a loading standard. (B) Sss1p expression, normalized to Actin expression, from each mutant was determined by quantitative Western blotting. Mutant Sss1p expression is presented graphically relative to Wt expression, with Wt expression set to 100%. (C) Yeast strain FKY 198 expressing Wt Sss1p and Sss1p mutants were grown in YEPD medium for 8 hours at 30°C, harvested, and whole cell lysates were prepared. Samples were separated by SDS-PAGE and analyzed by Western blotting using anti-Actin monoclonal and anti-Sss1p polyclonal antibodies.

Figure 15


kDa
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30
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68
9
45
68
45
HM
LM
cyt.
Wt
Vector
Sss1pTom5
Sss1pActA
Sss1pCTSa
Sss1pTMSa
Sss1pCTSwtVIV/AAA
Δ
-Actin
-Mir1p
-Sec63p
-x
-y
-Sec61p
*
-Sss1p
-Kar2p
-L3

Figure 15: Mutations in the TMS and CTS of Sss1p cause mislocalization of translocon proteins and interfere with ribosome binding to the ER

Yeast expressing Wt or mutant Sss1p were grown at 30°C in YEPD medium for 12 hours and were fractionated. Equal cell numbers of purified heavy membranes (HM), light membranes (LM) and cytosol (cyt.) were separated by SDS-PAGE and the localization of the ER proteins Sec63p, Sec61p, Sss1p and Kar2p, as well as the large ribosomal subunit L3, was determined by Western blotting. Actin was used as the cytosolic marker and the mitochondrial protein Mir1p was used as the HM marker. 'X' is an anti-Sec63p cross-reactive band. 'Y' is an anti-Sec61p cross-reactive band. The Sss1p cleavage product is identified as '*'. The symbols △, ▲, ● ● indicate the principal location of translocon components.

only a slight growth defect and properly localized a fraction of the translocon proteins to the LM fraction. There is also evidence that the growth defect observed in yeast expressing the Sss1pTMSa mutant may be caused by a defect in Sss1p function. Recall that the Sss1pK_5/T_5 mutant was predominantly mistargeted to the HM fraction (Figure 12D) yet still retained Wt growth (Figure 12B). The amount of Sss1p found in the LM fraction of Sss1pTMSa expressing yeast was much greater than that found in the LM fraction of yeast expressing the Sss1pK_5/T_5 mutant (compare LM fractions from Figure 15 – Sss1pTMSa and Figure 12D – Sss1pK_5/T_5). Yet yeast expressing the Sss1pTMSa mutant showed a growth defect. Therefore it is likely that the function of the Sss1pTMSa mutant is somewhat impaired. Recently, Mark Campbell (Andrews Lab) has demonstrated that all of the Sss1p mutants, with the exception of Sss1pTom5, are targeted to the ER membrane. This data confirms that most of the Sss1p mutants are in fact functionally defective.

3.9. LM localized Sss1p mutants behave like transmembrane proteins

To explore this apparent functional defect, purified ER membranes from several of the mutants (Sss1pCTSa, Sss1pTMSa, Sss1pCTSwtVIV/AAA) were subjected to various treatments to compare the membrane binding of the mutant proteins with that of Wt Sss1p (Figure 16). 250 or 500 A_{280} μU of membranes were treated with Buffer for 1 hour at 24°C as a control, 0.5 M KOAc for 30 minutes at 0°C, 2.5 M urea for 1 hour at 24°C, or 0.1 M carbonate pH 11.5 for 30 minutes at 0°C. Membranes were pelleted at 100,000*xg* to generate a supernatant (S) and pellet (P) fraction that contained primarily peripheral and integral membrane proteins respectively. The integral membrane protein Sec61p and the peripherally associated large ribosomal subunit L3 were used as controls.

As previously observed in Esnault et al., 1994, Wt Sss1p co-fractionated with Sec61p under all conditions. Sss1pCTSa, Sss1pTMSa and Sss1pCTSwtVIV/AAA mutant proteins, as well as the Sss1p cleavage product (*), also co-fractionated with Sec61p in the membrane pellet under all conditions, while the peripheral ribosomal protein L3 and a small amount of Sec61p were extracted by carbonate treatment (closed triangle). Our results demonstrate that Sss1p mutants associated with the LM fraction have similar membrane binding as Wt Sss1p and are therefore likely integrated into the ER membrane.

3.10. Mutations in the TMS or CTS of Sss1p disrupt ribosome binding to Sec61p

Since the Sss1p mutants analyzed were bound to the ER membrane like Wt, we tested the ability of both Sec61p and mutant Sss1p to bind to ribosomes (Figure 17A). In both the Sss1pTMSa and Sss1pCTSwtVIV/AAA mutant background a decrease in Sec61p co-fractionation with L3 was observed, with only 19% and 23% of Sec61p associated with ribosomes respectively (Figure 17B). The difference in Sec61p binding to ribosomes between these two mutants was not statistically significant. The Sss1pTMSa and Sss1pCTSwtVIV/AAA mutant proteins themselves were not associated with ribosomes, nor were the cleavage products (Figure 17A – *), while 32% of Wt Sss1p was

Figure 16

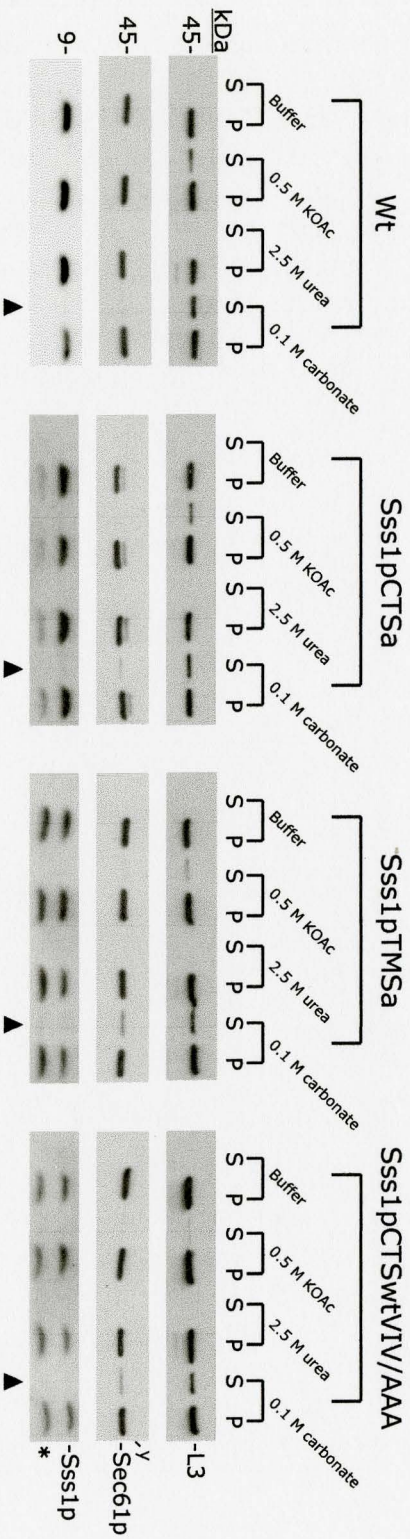
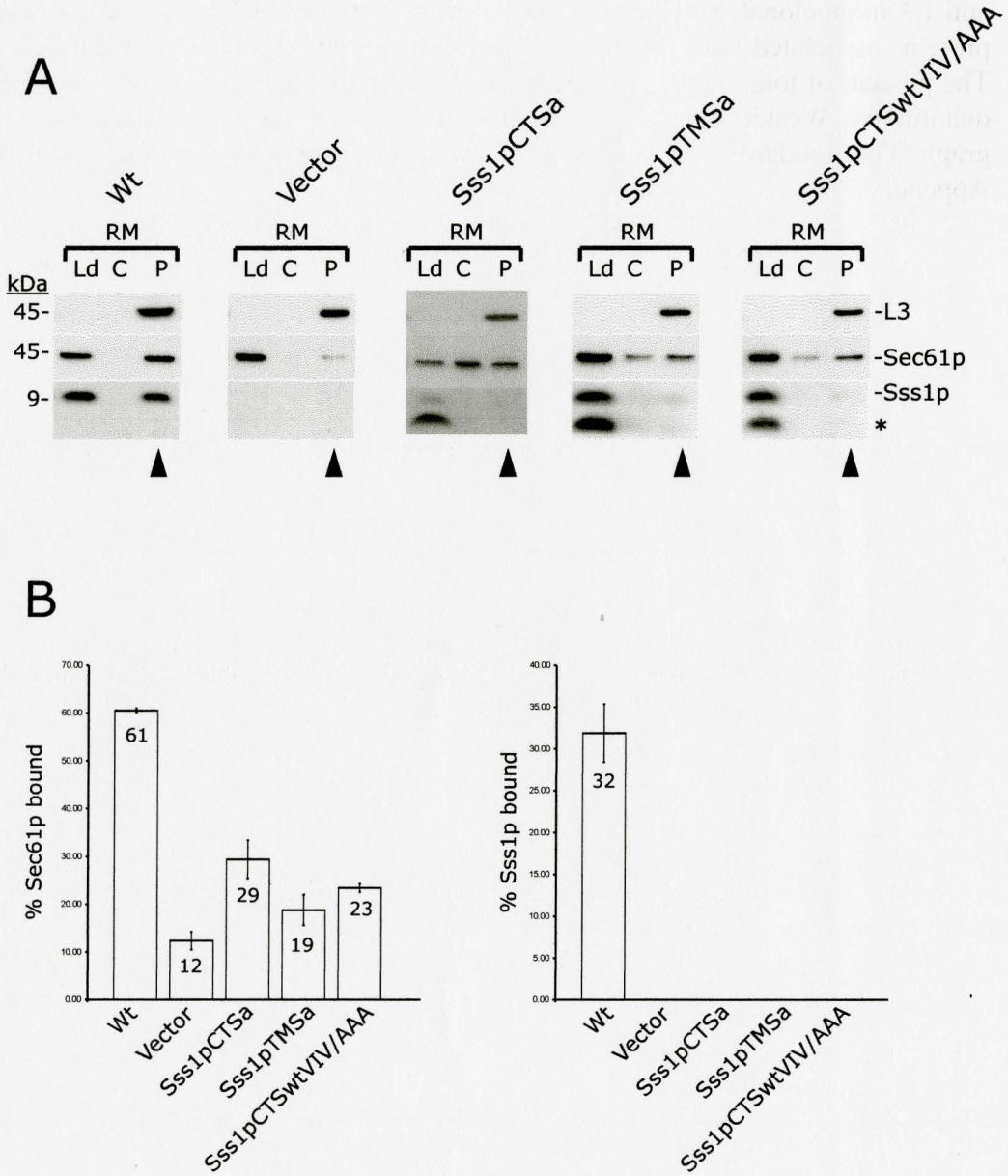

Wt
Sss1pCTSa
Sss1pTMSa
Sss1pCTSwtVIV/AAA
kDa
45-
9-
S P S P S P S P
Buffer
0.5 M KOAc
2.5 M urea
0.1 M carbonate
-L3
-Sec61p
-Sss1p
γ
*

Figure 16: Sss1p mutants in the LM fractions behave like transmembrane proteins

Purified ER membranes were subjected to various treatments to determine if the binding of Sss1p mutants was similar to Wt Sss1p. Equivalent A_{280} μUnits of rough microsomes were treated with Buffer (20 mM Tris·HCl pH 7.4, 250 mM sucrose, 5 mM $Mg(OAc)_2$) for 1 hour at 24°C, 0.5 M KOAc for 30 minutes at 0°C, 2.5 M urea for 1 hour at 24°C and 0.1 M carbonate pH 11.5 for 30 minutes at 0°C as indicated above the lanes. Membranes were centrifuged at 100,000*xg* to generate a supernatant (S) and membrane pellet (P) fraction). Equivalent volumes of supernatant and pellet fractions were separated by SDS-PAGE and analyzed by Western blotting using anti-L3 monoclonal, anti-Sec61p and anti-Sss1p polyclonal antibodies. 'Y' is an anti-Sec61p cross-reactive band. The Sss1p cleavage product is identified as '*'. ▲ indicates the location of peripherally associated membrane proteins.

Figure 17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Vector
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Sss1pTMSa
Sss1pCTSwtVIV/AAA
RM
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kDa
45-
45-
9-
-L3
-Sec61p
-Sss1p
*
B
% Sec61p bound
61
12
29
19
23
% Sss1p bound
32
Wt
Vector
Sss1pCTSa
Sss1pTMSa
Sss1pCTSwtVIV/AAA

Figure 17: Mutations in the TMS and CTS of Sss1p hinder ribosome binding to Sec61p and Sss1p

(A) Purified rough microsomes (RM) or puromycin/high salt treated rough microsomes (PKRM) were solubilized in 1% TX-100, layered over a 0.5 M sucrose cushion and centrifuged at 400,000*xg*. Equivalent volumes of load (Ld), cushion (C) and ribosome pellet (P) fractions were separated by SDS-PAGE and analyzed by Western blotting using anti-L3 monoclonal, anti-Sec61p and anti-Sss1p polyclonal antibodies. ▲ indicates the proteins associated with ribosomes. The Sss1p cleavage product is identified as '*'. (B) The percent of total Sec61p and Sss1p in the ribosome pellet fraction was determined by quantitative Western analysis using a Kodak Image Station and is presented in a bar graph. The standard curve for the anti-Sec61p and anti-Sss1p antibodies can be found in Appendix 1.

found in the ribosome pellet (Figure 17A - compare closed triangles, Figure 17B). The discrepancy between the percent of total Sss1p and Sec61p in the ribosome pellet (32% compared to 61%) is likely due to the membership of Sss1p in the Ssh1p complex, which has four-fold lower ribosome binding than the Sec61p complex (Prinz et al., 2000b). A surprising result was observed with the Sss1pCTSa mutant. In this mutant background, the amount of Sec61p found in the ribosome pellet was statistically larger than the Sss1pTMSa and Sss1pCTSwtVIV/AAA mutant (29% compared to 23% and 19% - Figure 17B).

The ability of the PCC to co-migrate with ribosomes was also analyzed in these Sss1p mutant backgrounds (Figure 18). Both the Sss1pTMSa and Sss1pCTSwtVIV/AAA solubilized RMs revealed a decline in Sec61p and Sss1p co-migration with L3, with the majority of Sec61p and Sss1p remaining at the top of the gradient with Sec63p (compare lanes 7–8, open arrows, with lanes 1-2). In addition, the observed PCC-ribosome complexes for Sss1pTMSa and Sss1pCTSwtVIV/AAA reproducibly migrated to fractions of lower sucrose density than Wt (Figure 18 – compare open arrows to closed arrows from Wt). Analysis of the Sss1pCTSa RMs also revealed a decline in Sec61p co-migration with L3. Unlike the Sss1pTMSa and Sss1pCTSwtVIV/AAA PCC-ribosome complexes, the Sss1pCTSa complexes migrated to the same fractions as Wt (compare closed arrows). As well, all of the cleavage products of the Sss1p mutants did not bind to ribosomes and remained at the top of the sucrose gradients.

Therefore mutations in the TMS or CTS of Sss1p decreased efficient Sec61p and Sss1p binding to ribosomes by approximately 40% and 30% respectively. However Sss1p mutants that contained the Wt TMS showed a statistically significant increase in Sec61p binding to ribosomes of up to 10%. Sss1p mutants with the ActA TMS altered the migration of the assembled ribosome-PCC complexes on a continuous sucrose gradient, while the ribosome-PCC complexes containing the Sss1p mutants with the Wt TMS migrated similarly to Wt complexes. The inability to form ribosome-PCC complexes likely contributed to the growth defect of yeast expressing these mutants, however it is not sufficient to fully explain the difference in growth between these mutants. For example, the Sss1pCTSwtVIV/AAA mutant showed similar ribosome binding to the Sss1pTMSa mutant, yet displayed 1000-fold lower growth. Similarly, the Sss1pCTSa mutant had 10% higher ribosome binding than the Sss1pTMSa mutant, yet had 100-fold lower growth, and was predominantly mislocalized to the HM fraction. There must therefore be some other unidentified defect(s) caused by these mutations that contribute to the decrease in yeast growth.

Figure 18

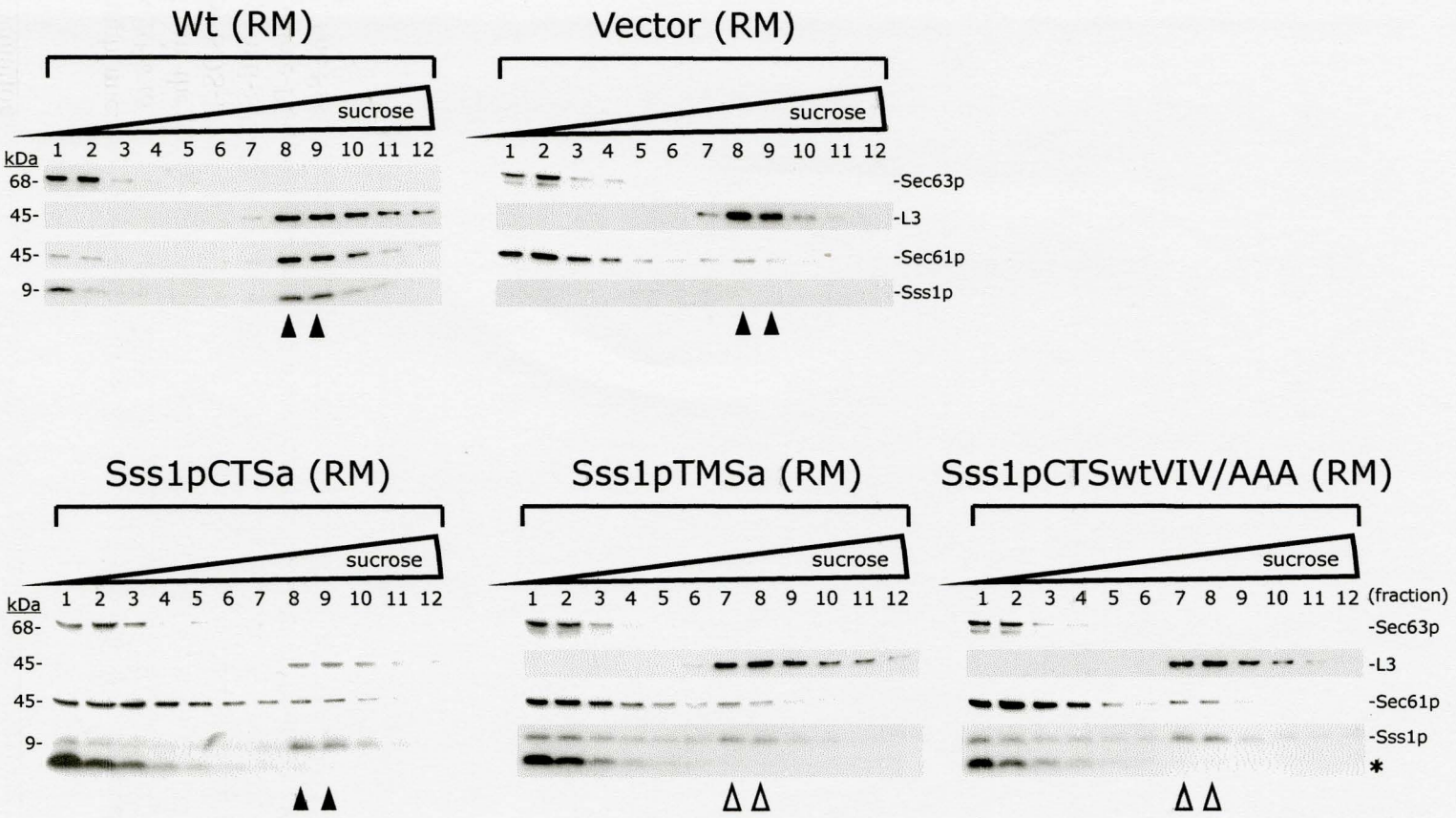
Wt (RM)
Vector (RM)
sucrose
kDa
1 2 3 4 5 6 7 8 9 10 11 12
68-
45-
45-
9-
-Sec63p
-L3
-Sec61p
-Sss1p
Sss1pCTSa (RM)
Sss1pTMSa (RM)
Sss1pCTSwtVIV/AAA (RM)
(fraction)
*

Figure 18: Mutations in the TMS and CTS of Sss1p reduce stable ribosome-PCC complex formation

Purified rough microsomes (RM) were solubilized in 1% TX-100, layered on top of a continuous 20% to 40% sucrose gradient and centrifuged at 230,000xg. Fractions were collected from the top of the gradient and equivalent volumes of each fraction were separated by SDS-PAGE and analyzed by Western blotting using anti-L3 monoclonal and anti-Sec63p, anti-Sec61p and anti-Sss1p polyclonal antibodies. ▲▲ indicates the location of Wt ribosome-PCC complexes. △△ indicates the location of non-Wt ribosome-PCC complexes. The Sss1p cleavage product is identified as '*'.

4. Discussion

The following conclusions can be drawn from the experimental results presented here. (1) Depletion of Sss1p leads to an apparently simultaneous block in co- and post-translational transport of proteins into the ER. (2) Over-expression of Sss1p leads to the accumulation of both Sss1p and Sec61p in HMs, an event that does not compromise yeast growth. (3) Sss1p contains an ER targeting signal located in the NTS, however disruption of the positive charges in the NTS does not abolish all targeting of Sss1p to the ER. Indeed, sufficient Sss1p ends up in the ER to preserve Wt growth. (4) We confirm that depleting the essential PCC components Sec61p or Sss1p completely inhibits cell growth. Depletion of either protein creates a block in co- and post-translational transport, causes severe mislocalization of translocon proteins to the HM fraction and inhibits efficient ribosome binding to Sec61p. (5) Substituting the TMS and/or CTS of Sss1p with those from other TA proteins led to growth defects in yeast, which were most severe when mutations were made to the CTS. There appears to be a positive correlation between the severity of the growth defect and the extent of mislocalization of translocon proteins to HMs. Even though all of the mutants examined demonstrated a decrease in ribosome binding to Sec61p, there was no correlation between the ribosome binding defect and the severity of the growth defect observed. (6) One mutant that is clearly localized to the ER, Sss1pTMSa, appears to be functionally defective. The defect is related to the block in Sec61p binding to ribosomes and may include other deficiencies not characterized in this study. The data presented here, supported by the preliminary results of Mark Campbell in our lab, lead us to speculate that mutations in the TMS or CTS of Sss1p: (1) inhibit Sss1p association with Sec61p and subsequently block efficient assembly of the PCC, (2) disrupt the signal sequence binding pocket of the PCC or (3) do both.

4.1. Effect of depletion and over-expression of Sss1p in yeast

The data in Figure 6B would suggest that the depletion of Sss1p simultaneously inhibits the translocation of co-translational (Kar2p) and post-translational (ppαF) substrates. As the experiment used two-hour time points, it is possible that the defect lies specifically with either co- or post-translational translocation. Since both pathways utilize the same PCC, a block in one pathway could block the second pathway. We observed that depletion of Sss1p inhibits efficient ribosome binding to Sec61p. Such a deficiency would lead to a defect in co-translational transport across the ER. It is therefore possible that the observed accumulation of co-translational substrates was directly or indirectly causing the defect in transport of post-translational substrates. However the data does not rule out the possibility that it is an initial defect in post-translational transport that is having a subsequent effect on ribosome binding to Sec61p and hence causing the defect in co-translational transport. A simple experiment, where Sss1p depletion time points are shortened to 30 minutes, could clarify this issue.

Recent studies have determined that the PCC exists as a trimer of heterotrimers, consisting of three Sec61p, Sbh1p and Sss1p molecules (Beckmann et al., 2001). Sss1p is also a component of a second PCC, the Ssh1p complex, composed of Ssh1p, Sbh2p and Sss1p (Finke et al., 1996). Therefore the regulation of Sss1p stoichiometry in the ER membrane may be important for the function of these channels. To address this

possibility, we over-expressed Sss1p in yeast and assayed their growth. As demonstrated in Figure 7, even when Sss1p is expressed at levels 20 times higher than Wt levels, yeast grow normally. The discovery that both Sec61p and Sss1p co-fractionate with mitochondria in the over-expressing strain was therefore surprising. Being a membrane associated protein, excess Sss1p would not be expected to remain soluble in the cytoplasm for long, and would likely form aggregates which could pellet with HMs at 20,000*xg*. It is also possible that the Sec61p found in the HM fraction were aggregates, as the over-expression of some proteins, in this case Sss1p, can cause the aggregation of others (Bence et al., 2001). To determine if the Sss1p and Sec61p found in the HM fraction are indeed aggregates, these membranes could be solubilized and subjected to gel filtration chromatography – large aggregates would be expected to elute in the excluded fraction. Alternatively, membrane floatation through sucrose could be employed – aggregates would not float with the membranes.

It may be that the over-expression of Sss1p does not lead to aggregation, but may change the structure of the ER such that it fractionates with HMs. Over-expression of the peroxisomal protein Pex15p in yeast led to the hyper-proliferation of ER membranes (Elgersma et al., 1997), which would be expected to change the fractionation pattern of the organelle. Similarly, over-expression of APX, a TA protein in plants, led to the proliferation of a sub-compartment of the ER (Mullen et al., 1999). If it were shown that ribosomes were also in the HM fraction of yeast over-expressing Sss1p (since they are not observed in Wt yeast), one could argue in favour of this theory since the aggregation of ribosomes would not be expected. Electron microscopy could be employed to determine if the ER structure has dramatically changed in the over-expressing strain.

Another interesting observation was the accumulation of a 7 kDa Sss1p cleavage product in the over-expressing strain. This cleavage occurs during the fractionation process, as it is not observed in yeast cells added directly to SDS-PAGE buffer. Wt Sss1p may be naturally susceptible to low levels of enzymatic cleavage during the fractionation process. This cleavage product may only be detectable by Western blotting when Sss1p is expressed at extremely high levels. However the degradation may be a result of the over-expression of Sss1p – under these conditions there may not be any 'free' translocons which Sss1p can be incorporated into, leaving 'bare' Sss1p molecules that may be more susceptible to enzymatic cleavage (Kalies et al., 1994; Murphy III et al., 1997; Potter & Nicchitta, 2000).

Three observations were made when we depleted yeast of either Sss1p or Sec61p. Sss1p depleted yeast appear to be capable of targeting and integrating newly synthesized Sss1p proteins independent of functional translocons (Figure 8). This observation is consistent with the current data that suggests TA proteins are targeted to the ER independent of the translocon (Kutay et al., 1995; Walter et al., 2001, Steel et al., 2002; Yabal et al., 2002). However, it is possible that Sss1p depletion was not complete in this experiment, and that there was still a small amount of Sss1p in the ER membranes that was below the detection limits of our assay. Depletion of translocation components is a slow process and appears to have wide-ranging effects on the cell (see below), further complicating the interpretation of this experiment. It may be more effective to use temperature sensitive (Ts) alleles of translocon components involved in both co- and post-

translational translocation to determine if Sss1p utilizes the translocon for its targeting and insertion into the ER membrane. At the non-permissive temperatures, translocation defects are observed between one and two hours (Deshaies & Schekman, 1987; Stirling et al., 1992).

Sss1p or Sec61p depleted yeast showed severe mislocalization of translocon proteins to HMs and also displayed a defect in ribosome binding to the ER membrane (Figure 9). It is possible that newly synthesized proteins destined for the ER are aggregating (and therefore pelleting with the HMs during fractionation) because of the translocation defect in the depleted yeast. Indeed, the presence of pKar2p, which should be cytoplasmic in translocation incompetent yeast, in the HM fraction of Sss1p and Sec61p depleted cells suggests these precursors are aggregating. If the translocon components found in the HM fraction are aggregates, the implications for Sss1p targeting to the ER could be profound, given that in Sec61p depleted cells Sss1p is also found in the HM fraction. If Sss1p does not require the translocon to be targeted to the ER, one would have expected Sss1p to be localized to the LM fraction in the Secc61p-depleted strain. Instead, Sss1p, like Sec61p, may require the translocon for its proper targeting and integration into the ER membrane, consistent with the model presented by Abell et al., 2002. However it is also possible that an unidentified protein(s) ('Protein X') involved in targeting Sss1p relies on the translocon to be properly associated with the ER membrane. Depletion of Sec61p would inhibit the targeting of 'Protein X' and would subsequently block the targeting of Sss1p to the ER.

There is yet another possible factor that could contribute to these observations. A paper published by the Rapoport lab has shown that a 33% decrease in ribosome binding to Sec61p, along with a translocation defect, disrupts the structure of the ER in yeast (Prinz et al., 2000c). Immunofluorescense data from that paper demonstrated that the disrupted ER co-localized with mitochondria. It is then possible that the ER in Sss1p and Sec61p depleted cells has at least partially collapsed onto the mitochondria and therefore pellets with HMs during fractionation. This opens up the possibility that Sss1p is targeted to the ER independent of the translocon, but is found in the HM fraction because depletion of Sec61p leads to ER collapse onto mitochondria. In addition, since ribosome binding is not completely lost in the depleted yeast, ER collapse onto mitochondria would be consistent with our discovery of ribosomes in the HM fraction of Sss1p depleted yeast but not in Wt yeast (Figure 9).

Depletion of Sss1p was found to disrupt ribosome-PCC complexes by decreasing the binding of ribosomes to Sec61p by approximately 80% compared to Wt (Figure 10). Therefore it appears that Sss1p is essential for ribosome binding to Sec61p. One may argue that the decrease in Sec61p pelleting in this assay is due to the fact that there are fewer ribosomes on Sss1p depleted membranes than on Wt membranes (Figure 9). For this argument to be valid it would be necessary that both Wt and depleted ER contain equal amounts of Sec61p. Figure 9 clearly demonstrates that this is not the case. In addition, the pelleting assay was internally controlled, as we analyzed only the percent of total Sec61p in the ribosome pellet from purified ER. Under these conditions, were Sss1p not essential for Sec61p binding to ribosomes, we would not expect to see any difference in the percent of total Sec61p found in the ribosome pellet between Wt and Sss1p

depleted cells. Although one may criticize that the data in Figure 9 was generated from yeast depleted of Sss1p for 12 hours, while ER membranes used in the ribosome pelleting assay were purified after 8 hours of depletion, we do not feel that this would influence our interpretation. For this criticism to be valid there must be reason to believe that both Wt and Sss1p depleted ER would contain approximately equal amounts of Sec61p after 8 hours of depletion. It is unlikely that this is the case given that the translocation defect occurs after 4 hours of Sss1p depletion. Since Sec61p is turned over more rapidly when Sss1p is depleted (Esnault et al., 1994; Biederer et al., 1996) and newly synthesized Sec61p requires a functional translocon to be integrated into the ER membrane, it is likely that Sss1p depleted ER contain less Sec61p than Wt ER after 8 hours.

4.2. ER targeting signal(s) in the TA of Sss1p

Disruption of the putative ER targeting signal in the NTS did target Sss1p away from the ER to HMs, however the mistargeting was incomplete (Figure 12). A small amount of Sss1p, sufficient to sustain Wt growth, ended up in the ER, suggesting that the disruption of the signal was incomplete, that there may be other ER targeting signal(s) in the TMS and/or CTS not identified in this study, or some Sss1p is targeted to the ER membrane by default. A more drastic mutation in the NTS, where the entire NTS was replaced with that from Tom22p, was not tolerated (Mark Campbell, personal communication). This mutant could not be stably expressed in yeast, suggesting that the NTS may influence the global folding of the protein, or may also have a role in Sss1p function, which, when not present, leads to the rapid degradation of the protein.

4.3. Effect of mutations in the TMS and CTS of Sss1p

Replacing the TMS and CTS of Sss1p with those from the TA proteins Tom5p or ActA caused growth defects in yeast. Those mutants that lacked the Wt CTS showed the most severe growth defects (Figure 13). There was also a correlation between diminished yeast growth and mislocalization of translocon proteins to HMs (Figure 15). The Sss1p mutants carried targeting signals that have been shown to be sufficient to target proteins to the mitochondria in mammalian cells (Zhu et al., 1996; Horie et al., 2002). These mutants may therefore have been mislocalized to the mitochondria in yeast. The mistargeting of Sss1p would be expected to cause defects in co- and post-translational translocation (likely leading to the aggregation of ER-destined membrane proteins which would pellet with HMs), and inhibit ribosome binding to Sec61p (consistent with our Sss1p depletion data) which may lead to ER collapse onto mitochondria.

Mistargeting could be confirmed by quantitatively comparing the distribution of total Sss1p between the LM and HM fractions in yeast that express Wt Sss1p (which is predominantly in the LM fraction) to yeast that co-express Wt and mutant Sss1p. Under these conditions, if the mutant protein is being mislocalized the total amount of Sss1p in the LM fraction would decrease, while that found in the HM fraction would increase. Another more involved experiment could be done where Sss1p mutants are myc-tagged and expressed in Wt yeast. Assuming the tag does not interfere with Sss1p targeting or function, the localization of mutant Sss1p proteins could be determined by fractionation and Western blotting with an anti-myc monoclonal antibody.

However the data also support the hypothesis that the Sss1p mutants were properly targeted to the ER but were functionally defective. Defective Sss1p molecules would create a translocation defect, and could subsequently cause aggregation of ER destined translocon components, inhibit ribosome binding to Sec61p and lead to ER collapse onto mitochondria. Data generated by Mark Campbell in our lab suggest that this hypothesis may be correct. He has fractionated yeast co-expressing Wt Sss1p with all of the Sss1p mutants from this study and has shown that mutant Sss1p proteins are predominantly in the LM fraction, demonstrating that most of the Sss1p mutants (with the exception of the Sss1pTom5 mutant, which is found in the HM fraction) are in fact being targeted to the ER.

The data presented in Figure 16 suggest that ER associated mutant Sss1p binds to the membrane like Wt. The fractionation data presented in Figure 15 show translocon proteins predominantly in the HM fraction after 12 hours growth in YEPD medium (with the exception of Sss1pTMSa). Figure 14 shows severe co- and post-translational defects in yeast expressing Sss1p mutants. Figures 17 and 18 show a defect in ribosome binding to Sec61p after 8 hours. Taken together, these observations (along with those of Mark Campbell) lead to the following conclusions. Since most of the Sss1p mutants are targeted to the ER, the observed defect in co- and post-translational translocation indicate that the Sss1p mutants suffer from a functional deficiency. Therefore the apparent mislocalization of translocon components to HMs after 12 hours of growth in YEPD medium is likely attributable to a combination of two events. (1) Newly synthesized translocon proteins that cannot be targeted due to the translocation defect subsequently aggregate and are pelleted with HMs during the fractionation process; (2) The collapse of the ER onto mitochondria (HM fraction) caused by the ribosome binding and transport defect cause the ER to co-fractionate with mitochondria.

A quantitative fractionation experiment could help confirm if ER collapse is contributing to the mislocalization of translocon components. Yeast expressing the Ts *sec61-3* allele as the sole copy of Sec61p in the cell show translocation defects at the non-permissive temperature yet have Wt ribosome binding to Sec61p (Prinz et al., 2000c). Using this strain we could determine the location of ER proteins by fractionation, and compare the amount of translocon components in the HM fraction to the amount found in the yeast analyzed in this study. Those translocon components found in the HM fraction in the *sec61-3* strain would likely be aggregates, since the yeast have near normal ER structure (Prinz et al., 2000c), while those found in the Sss1p mutant strains would be caused by a combination of aggregation and ER collapse. If the ER is indeed collapsing, then the total amount of translocon proteins found in HMs should be statistically higher in the Sss1p mutant strains than in the *sec61-3* strain.

How do we reconcile the conclusions made above with the results obtained with the Sss1pTMSa mutant? Like the other mutant strains, yeast expressing the Sss1pTMSa protein showed both severe co- and post-translational transport and ribosome binding defects. Yet these same yeast had growth near Wt levels and showed incomplete mislocalization of translocon proteins to HMs. Assuming our interpretation of the Rapoport group's data is correct, it is likely that the ER is collapsing onto mitochondria in

the Sss1pTMSa expressing yeast. However, what may differ in this mutant background is the kinetics of the translocation defect. Our Western blot data (Figure 14) only indicate that yeast expressing Sss1p mutants suffer a block in co- and post-translational transport. The data does not show that the block is complete (the fact that the yeast show some growth indicate it is not) nor can it establish differences in the kinetics of the translocation defect between mutants. It is therefore possible that the defect suffered by the Sss1pTMSa mutant is less severe than the defect in the other mutants, such that it can properly target and insert a greater proportion of the ER membrane proteins analyzed, resulting in its superior growth. This theory can be tested experimentally through the use of pulse-chase analysis. Yeast expressing the Sss1pTMSa mutant would be expected to have lower rates of precursor accumulation than yeast expressing the other Sss1p mutants. It must be noted that any targeting that does occur is likely SRP-independent given the observed defect in ribosome binding to Sec61p – this is not detrimental to the hypothesis as yeast are capable of targeting co-translational substrates independent of SRP (Waters and Blobel, 1986).

4.4. Possible functions of Sss1p

Given the results of Mark Campbell's targeting experiments which show that most Sss1p mutants are targeted to the ER membrane, it is clear that the specific amino acid sequence of the TMS and CTS of Sss1p are important for Sss1p function. The importance of the TMS and CTS for Sss1p function is not very surprising, given that the TMS and CTS have 44% and 64% amino acid identity respectively, to the TMS and CTS of the mammalian homolog Sec61γ.

The data presented in Figures 17 and 18 demonstrate that the TMS and CTS of Sss1p are involved in ribosome binding to Sec61p. A mutant which carries the Wt TMS but contains the CTS from ActA (Sss1pCTSa), showed a small yet statistically significant increase in ribosome binding to Sec61p than a mutant which carried the ActA TMS and the Wt CTS (Sss1pTMSa). Similarly, ribosome-PCC complexes containing the Sss1pCTSa mutant, though diminished in number (see Figure 15), showed the same migration pattern as Wt complexes on a continuous sucrose gradient (Figure 18). In contrast, complexes that contained the Sss1pTMSa mutant showed a slightly different migration pattern than Wt, suggesting that these complexes may have a different composition or configuration than Wt complexes. A mutant where both the TMS and CTS were mutated (Sss1pCTSwtVIV/AAA) showed similar results as the Sss1pTMSa mutant. Therefore the Wt TMS appears to play a more pivotal role than the CTS in this function.

It is curious that purified ER containing the Sss1pCTSa mutant protein showed higher ribosome binding to Sec61p than those with the Sss1pTMSa mutant protein, when yeast that express this Sss1pCTSa have 100-fold lower growth than yeast expressing the Sss1pTMSa protein. One could speculate, based on the conclusions drawn above, that yeast expressing an Sss1p mutant lacking a Wt CTS have a greater kinetic defect in translocation that is unrelated to ribosome binding. Alternatively, the lack of a Wt CTS may cause another functional defect not identified in this study. What could this

unidentified function be? The CTS of Sss1p may be important for the ER associated degradation (ERAD) pathway, where proteins are 'retrotranslocated' through the PCC from the ER lumen to the cytosol for degradation (Pilon et al., 1997). The *sec61-3* mutant, which maps to a point mutation in lumenal loop 7 of the protein (Wilkinson et al., 1997), is defective for the export of proteins from the lumen to the cytosol *in vitro* (Pilon et al., 1997). Since Sss1p has been shown to interact with this region of Sec61p (Wilkinson et al., 1997), the CTS may help facilitate the retrograde transport of proteins. Therefore, a kinetic defect in translocation or a defect in ERAD, combined with the observed decrease in ribosome binding, may explain the diminished growth of yeast expressing Sss1p mutants lacking a Wt CTS.

It is therefore clear that the TMS and CTS of Sss1p play an important role in the function of the protein. Our data demonstrates that Sss1p plays an essential role in efficient ribosome binding to Sec61p during co-translational transport and is necessary for post-translational translocation. Numerous functions for Sss1p have been suggested in the literature. Sss1p may interact with the SRP-SR complex, and help facilitate the transfer of the RNC to the translocon. This role would be consistent with Sss1p being a part of both PCC complexes in yeast, but would only explain the block in co-translational transport observed in this study.

Sss1p could recruit Sec63p to the PCC; mutations in Sss1p or depletion of the protein would explain the block in both co- and post-translational targeting, since Sec63p is essential for both pathways in yeast. However this role for Sss1p does not explain the observed defect in ribosome binding to Sec61p in this study, as Sec63p has not been implicated in this process. It has also been suggested that Sss1p may act as a 'plug' for the PCC to maintain the permeability barrier between the ER and cytosol. Though mammalian studies demonstrate that the ribosome and BiP perform this function, the maintenance of the permeability barrier in yeast has not been studied. Our data does not rule out the possibility of this function for Sss1p, however the depletion of the channel plug would not be expected to interfere with co- and post-translational transport.

Plath et al., 1998, have suggested that Sss1p acts as a place-holder for signal sequences, as they have observed chemical cross-links between signal sequences and TMS 2 and 7 of Sec61p – TMS7 being a region that Sss1p is observed to be in close proximity (Wilkinson et al., 1997). Although our data does not rule out this possible function, there are problems with this theory. Sss1p has not been demonstrated to be near TMS2 of Sec61p, and TMS2 can be deleted without compromising translocon function (Wilkinson et al., 2000). Plath further suggests that due to the essential promiscuous properties of the signal sequence binding site (given the plurality of signal peptides) the specific amino acid sequence of the TA of Sss1p would not be important. To the contrary, we have shown that the specific amino acid sequence of the TA of Sss1p is important. Finally, it is not clear why the depletion or deletion of a signal sequence place-holder would lead to a block in co- and post-translational translocation and the subsequent death of the cell.

We suggest two potentially over-lapping models of Sss1p function that are consistent with what is currently known about Sss1p. (1) Sss1p is an essential contributor

to the signal sequence binding site and/or (2) Sss1p is a structural protein that is essential for the integrity of the PCC.

If Sss1p helps form the signal sequence binding pocket, mutations in or depletion of Sss1p could cause blocks in both co- and post-translational translocation. The signal sequence must be recognized by the signal sequence binding site in the translocon to initiate tight binding of the ribosome to the PCC in co-translational transport (Jungnickel & Rapoport, 1995). If Sss1p is mutated or absent this event may not occur and would therefore inhibit tight binding of ribosomes to Sec61p, subsequently blocking co-translational transport. In addition, the signal sequence binding site is essential for post-translational targeting and transport (Feldheim & Schekman, 1994; Lyman & Schekman, 1997; Pilon et al., 1998; Plath et al., 1998; Dunnwald et al., 1999), therefore similar mutations in Sss1p that would block co-translational translocation would be expected to block post-translational transport as well.

Sss1p may also be a structural protein, holding Sec61p, and potentially Ssh1p, monomers together to form the functional PCC. Mutations in or depletion of Sss1p could cause the disassembly of the PCC and disrupt both co- and post-translational targeting. There is experimental evidence that supports this hypothesis. First, Sss1p has been shown to physically stabilize Ts Sec61p mutants (Esnault et al., 1994; Wilkinson et al., 1997) and block degradation of Sec61p by the proteasome (Biederer et al., 1996). Biederer's model of Sec61p degradation states that only Sec61p monomers are marked for degradation, suggesting that Sss1p rescues Sec61p by keeping it in a polymeric state, such as the Sec61p-Sbh1p-Sss1p trimer, or the trimer of heterotrimers forming the PCC. Our data also hints that mutations in the TMS and/or CTS of Sss1p lead to the dissociation of Sec61p and Sss1p. We consistently observed an anti-Sec61p cross-reactive band that migrated slightly above Sec61p only in yeast that lacked Sss1p or expressed Sss1p mutants. Sec61p does not contain a cleavable signal sequence (it is targeted to the ER by TMS1), therefore this cross-reactive band cannot be a Sec61p precursor. Since ubiquitin is 76 amino acids in length, it is possible that this cross-reactive band is ubiquitinated Sec61p. Western blotting or immunoprecipitation with an anti-ubiquitin antibody may determine if Sec61p is indeed being ubiquitinated under the conditions in this study.

Second, Wilkinson et al., 1997, only observed Sec61p-Sss1p chemical cross-links, and never observed cross-links between Sec61p monomers, when they analyzed the physical interaction of Sss1p with Sec61p. Third, ribosomes can only bind to mammalian Sec61p in the polymeric state (Raden et al., 2000). Assuming that ribosome binding to Sec61p is conserved between mammals and yeast, our observation that mutations in the TMS/CTS or the depletion of Sss1p diminish efficient ribosome binding to Sec61p suggest that under these conditions Sec61p is not effectively oligomerized.

The described role(s) for Sss1p may also explain the high susceptibility of Sss1p mutants to proteolytic cleavage, forming a membrane bound 7 kDa cleavage product, observed during the fractionation process in this study. The inability to form a complex with Sec61p and the lack of protection by bound ribosomes would likely make these mutant proteins more accessible to proteases (Kalies et al., 1994; Murphy III et al., 1997; Potter & Nicchitta, 2000). It is possible to experimentally determine the necessity of

Sss1p for oligomerization of the PCC. Gel filtration chromatography was used to determine the binding partners of Sec63p in yeast (Brodsky & Schekman, 1993). The same experiment is now being employed in our lab to assay the polymeric state of Sec61p from purified ER in Wt yeast, Sss1p depleted yeast and yeast expressing mutant Sss1p proteins.

Bibliography

Abell, B.M., Jung, M., Oliver, J.D., Knight, B.C., Tyedmers, J., Zimmermann, R., High, S., "Tail-anchored and signal-anchored proteins utilize overlapping pathways during membrane insertion" (2002) Journal of Biological Chemistry Papers in Press, Manuscript M209968200.

Allen, R., Egan, B., Gabriel, K., Beilharz, T., Lithgow, T., "A conserved proline residue is present in the transmembrane-spanning domain of Tom7 and other tail-anchored protein subunits of the TOM translocase" (1999) 514 FEBS Letters 347.

Anderson, D.J., Mostov, K.E., Blobel, G., "Mechanisms of integration of de novo-synthesized polypeptides into membranes: Signal-recognition particle is required for integration into microsomal membranes of calcium ATPase and lens MP26 but not of cytochrome b_5" (1983) 80 PNAS 7249.

Antonin W. & Meyer H.A., Hartmann, E., "Interactions between Spc2p and Other Components of the Endoplasmic Reticulum Translocation Sites of the Yeast *Saccharomyces cerevisiae*" (2000) 275 Journal of Biological Chemistry 34068.

Bacher, G., Pool, M., Dobberstein, B., "The Ribosome Regulates the GTPase of the β-subunit of the Signal Recognition Particle Receptor" (1999) 146 Journal of Cell Biology 723.

Becker, J., Walter, W., Yan, W., Craig, E.A., "Functional Interaction of Cytosolic hsp70 and a DnaJ-Related Protein, Ydj1p, in Protein Translocation *In vivo*" (1996) 16 Molecular and Cellular Biology 4378.

Beckmann, R., Spahn, C.M.T., Eswar, N., Helmers, J., Penczek, P.A., Sali, A., Frank, J., Blobel, G., "Architecture of the Protein-Conducting Channel Associated with the Translating 80S Ribosome" (2001) 107 Cell 361.

Beilharz, T., Egan, B., Silver, P.A., Hofmann, K., Lithgow, T., "Bipartite signals mediate subcellular targeting of tail-anchored membrane proteins in *Saccharomyces cerevisiae*" (2003) Journal of Biological Chemistry Papers in Press, Manuscript M212725200.

Bence, N.F., Sampat, R.M., Kopito, R.R., "Impairment of the Ubiquitin-Proteasome System by Protein Aggregation" (2001) 292 Science 1552.

Beswick, V., Brodsky, J.S., Kepes, F., Neumann, J., Sanson, A., Garrigos, M., "Expression, Purification, and Characterization of Sss1p, and Essential Component of the Yeast Sec61p Protein Translocation Apparatus" (1998) 13 Protein Expression and Purification 423.

Biederer, T., Volkwein, C., Sommer, T., "Degradation of subunits of the Sec61p complex, an integral component of the ER membrane, by the ubiquitin-proteasome pathway" (1996) 15 EMBO 2069.

Boisrame, A., Chasles, M., Babour, A., Beckerich, J.M., Gaillardin, C., "Sbh1p, a subunit of the Sec61 translocon, interacts with the chaperone calnexin in the yeast *Yarrowia lipolytica*" (2002) 115 Journal of Cell Science 4947.

Borgese, N., Gazzoni, I., Barber, M., Colombo, S., Pedrazzini, E., "Targeting of a Tail-anchored Protein to Endoplasmic Reticulum and Mitochondrial Outer Membrane by Independent but Competing Pathways" (2001) 12 Molecular Biology of the Cell 2482.

Brizzio, V., Khalfan, W., Huddler, D., Beh, C.T., Andersen, S.S., Latterich, M., Rose, M.D., "Genetic interactions between KAR7/SEC71, KAR8/JEM1, KAR5, and KAR2 during nuclear fusion in *Saccharomyces cerevisiae*" (1999) 10 Molecular Biology of the Cell 609.

Brodsky, J.L., Hamamoto, S., Feldheim, D., Schekman, R., "Reconstitution of Protein Translocation from Solubilized Yeast Membranes Reveals Topologically Distinct Roles for BiP and Cytosolic Hsc70" (1993) 120 Journal of Cell Biology 95.

Brodsky, J.L. & Schekman, R., "A Sec63p-BiP Complex from Yeast Is Required for Protein Translocation in a Reconstituted Proteoliposome" (1993) 123 Journal of Cell Biology 1355.

Brodsky, J.L., Goeckeler, J., Schekman, R., "BiP and Sec63- are required for both co- and posttranslational protein translocation into the yeast endoplasmic reticulum" (1995) 92 PNAS 9643.

Brodsky, J.L., Bauerle, M., Horst, M., McClellan, A.J., "Mitochondrial Hsp70 cannot replace BiP in driving protein translocation into the yeast endoplasmic reticulum" (1998) 435 FEBS Letters 183.

Brown, J., Hann, B., Medzihradszky, K., Niwa, M., Burlingame, A.L., Walter, P., "Subunits of the *Saccharomyces cerevisiae* signal recognition particle required for its functional expression" (1994) 13 EMBO 4390.

Caplan, A.J., Cyr, D.M., Douglas, M.G., "YDJ1p Facilitates Polypeptide Translocation across Different Intracellular Membranes by a Conserved Mechanism" (1992) 71 Cell 1143.

Chirico, W.J., Waters, M.G., Blobel, G., "70K heat shock related proteins stimulate protein translocation into microsomes" (1988) 332 Nature 805.

Connolly, T. & Gilmore, R., "The Signal Recognition Particle Receptor Mediates the GTP-Dependent Displacement of SRP from the Signal Sequence of the Nascent Polypeptide" (1989) 57 Cell 599.

Connolly, T., Rapiejko, P.J., Gilmore, R., "Requirement of GTP Hydrolysis for Dissociation of the Signal Recognition Particle form Its Receptor" (1991) 252 Science 1171.

Corsi, A.K. & Schekman, R., "The Lumenal Domain of Sec63p Stimulates the ATPase Activity of BiP and Mediates BiP Recruitment to the Translocon in *Saccharomyces cerevisiae*" (1997) 137 Journal of Cell Biology 1483.

Craven, R.A., Egerton, M., Stirling, C.J., "A novel Hsp70 of the yeast ER lumen is required for the efficient translocation of a number of protein precursors" (1996) 15 EMBO 2640.

Crowley, K.S., Liao, S., Worrell, V.E., Reinhart, G.D., Johnson, A.E., "Secretory Proteins Move through the Endoplasmic Reticulum Membrane via an Aqueous, Gated Pore" (1994) 78 Cell 461.

Crowley, K.S., Reinhart, G.D., Johnson, A.E., "The Signal Sequence Moves through a Ribosomal Tunnel into a Noncytoplasmic Aqueous Environment at the ER Membrane Early in Translocation" (1993) 73 Cell 1101.

Dembowski, M., Kunkele, KP., Nargang, F.E., Neupert, W., Rapoport, D., "Assembly of Tom6 and Tom7 into the TOM Core Complex of *Neurospora crassa*" (2001) 276 Journal of Biological Chemistry 17679.

Deshaies, R.J. & Schekman, R., "Structural and Functional Dissection of Sec62p, a Membrane-Bound Component of the Yeast Endoplasmic Reticulum Protein Import Machinery" (1990) 10 Molecular and Cellular Biology 6024.

Deshaies, R.J., Sanders, S.L., Feldheim, D.A., Schekman, R., "Assembly of yeast Sec proteins involved in translocation into the endoplasmic reticulum into a membrane-bound multisubunit complex" (1991) 349 Nature 806.

Dietmeier, K. & Honlinger, A., Bomer, U., Dekker, P.J.T., Eckerskorn, C., Lottspeich, F., Kubrich, M., Pfanner, N., "Tom5 functionally links mitochondrial preprotein receptors to the general import pore" (1997) 388 Nature 195.

Do, H., Falcone, D., Lin, J., Andrews, D.W., Johnson, A.E., "The Cotranslational Integration of Membrane Proteins into the Phospholipid Bilayer Is a Multistep Process" (1996) 85 Cell 369.

Dudek, J., Volkmer, J., Bies, C., Guth, S., Muller, A., Lerner, M., Feick, P., Schafer, KH., Morgenstern, E., Hennessy, F., Blatch, G.L., Janoscheck, K., Heim, N., Scholtes, P., Frien, M., Nastainczyk, W., Zimmermann, R., "A novel type of co-chaperone mediates transmembrane recruitment of DnaK-like chaperones to ribosomes" (2002) 21 EMBO 2958.

Dunnwald, M., Varshavsky, A., Johnsson, N., "Detection of Transient *In vivo* Interactions between Substrate and Transporter during Protein Translocation into the Endoplasmic Reticulum" (1999) 10 Molecular Biology of the Cell 329.

Egan, B., Beilharz, T., George, R., Isenmann, S., Gratzer, S., Wattenberg, B., Lithgow, T., "Targeting of tail-anchored proteins to yeast mitochondria *in vivo*" (1999) 451 FEBS Letters 243.

Elgersma, Y., Kwast, L., van den Berg, M., Snyder, W.B., Distel, B., Subramani, S., Tabak, H., "Overexpression of Pex15p, a phosphorylated peroxisomal integral membrane protein required for peroxisome assembly in *S. cerevisiae*, causes proliferation of the endoplasmic reticulum membrane" (1997) 16 EMBO 7326.

Esnault, Y., Blondel, M.O., Deshaies, R.J., Schekman, R., Kepes, F., "The yeast *SSS1* gene is essential for secretory protein translocation and encodes a conserved protein of the endoplasmic reticulum" (1993) 12 EMBO 4083.

Esnault, Y., Feldheim, D., Blondel, M.O., Schekman, R., Kepes, F., "*SSS1* Encodes a Stabilizing Component of the Sec61 Subcomplex of the Yeast Protein Translocation Apparatus" (1994) 269 Journal of Biological Chemistry 27478.

Fang, H. & Green, N., "Nonlethal *sec71-1* and *sec72-1* Mutations Eliminate Proteins Associated with the Sec63p-BiP Complex from *S. cerevisiae*" (1994) 5 Molecular Biology of the Cell 933.

Feldheim, D., Rothblatt, J., Schekman, R., "Topology and Functional Domains of Sec63p, and Endoplasmic Reticulum Membrane Protein Required for Secretory Protein Translocation" (1992) 12 Molecular and Cellular Biology 3288.

Feldheim, D., Yoshimura, K., Admon, A., Schekman, R., "Stuctural and Functional Characterization of Sec66p, a New Subunit of the Polypeptide Translocation Apparatus in the Yeast Endoplasmic Reticulum" (1993) 4 Molecular Biology of the Cell 931.

Feldheim, D., & Schekman, R., "Sec72p Contributes to the Selective Recognition of Signal Peptides by the Secretory Polypeptide Translocation Complex" (1994) 126 Journal of Cell Biology 935.

Finke, K., Plath, K., Panzner, S., Prehn, S., Rapoport, T.A., Hartmann, E., Sommer, T., "A second trimeric complex containing homologs of the Sec61p complex functions in protein transport across the ER membrane of *S. cerevisiae*" (1996) 15 EMBO 1482.

Fulga, T.A., Sinning, I., Dobberstein, B., Pool, M.R., "SRβ coordinates signal sequence release from SRP with ribosome binding to the translocon" (2001) 20 EMBO 2338.

George, R., Beddoe, T., Landi, K., Lithgow, T., "The yeast nascent polypeptide-associated complex initiates protein targeting to mitochondria *in vivo*" (1998) 95 PNAS 2296.

Gorlich, D., Hartmann, E., Prehn, S., Rapoport, T.A., "A protein of the endoplasmic reticulum involved early in polypeptide translocation" (1992a) 357 Nature 47.

Gorlich, D., Prehn, S., Hartmann, E., Kalies, KU., Rapoport, T.A., "A Mammalian Homolog of SEC61p and SECYp Is Associated with Ribosomes and Nascent Polypeptides during Translocation" (1992b) 71 Cell 489.

Gorlich, D. & Rapoport, T.A., "Protein Translocation into Proteoliposomes Reconstituted from Purified Components of the Endoplasmic Reticulum Membrane" (1993) 75 Cell 615.

Haigh, N.G. & Johnson, A.E., "A new role for BiP: closing aqueous translocon pore during protein integration into the ER membrane" (2002) 156 Journal of Cell Biology 261.

Hamman, B.D., Chen, JC., Johnson, E.E., Johnson, A.E., "The Aqueous Pore through the Translocon Has a Diameter of 40-60 Angstroms During Cotranslational Protein Translocation at the ER Membrane" (1997) 89 Cell 535.

Hamman, B.D., Hendershot, L.M., Johnson, A.E., "BiP Maintains the Permeability Barrier of the ER Membrane by Sealing the Lumenal End of the Translocon Pore before and Early in Translocation" (1998) 92 Cell 747.

Hamilton, T.G., Norris, T.B., Tsuruda, P.R., Flynn, G.C., "Cer1p functions as a molecular chaperone in the endoplasmic reticulum of *Saccharomyces cerevisiae*" (1999) 19 Molecular and Cellular Biology 5298.

Hanein, D., Matlack, K.E.S., Jungnickel, B., Plath, K., Kalies, KU., Miller, K.R., Rapoport, T.A., Akey, C.W., "Oligomeric Rings of the Sec61p Complex Induced by Ligands Required for Protein Translocation" (1996) 87 Cell 721.

Hann, B.C., Poritz, M.A., Walter, P., "*Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* Contain a Homologue of the 54-kD Subunit of The Signal Recognition Particle That in *S. cerevisiae* is Essential for Growth" (1989) 109 Journal of Cell Biology 3223.

Hann, B. & Walter, P., "The Signal Recognition Particle in *S. cerevisiae*" (1991) 67 Cell 131.

Hann, B., Stirling, C.J., Walter, P., "*SEC65* gene product is a subunit of the yeast signal recognition particle required for its integrity" (1992) 356 Nature 532.

Hartmann, E., Sommer, T., Prehn, S., Gorlich, D., Jentsch, S., Rapoport, T., "Evolutionary conservation of components of the protein translocation complex" (1994) 367 Nature 654.

Hauser, S., Bacher, G., Dobberstein, B., Lutcke, H., "A complex of the signal sequence binding protein and the SRP RNA promotes translocation of nascent proteins" (1995) 14 EMBO 5485.

Heinrich, S.U., Mothes, W., Brunner, J., Rapoport, T.A., "The Sec61p Complex Mediates the Integration of a Membrane Protein by Allowing Lipid Partitioning of the Transmembrane Domain" (2000) 102 Cell 233.

Heritage, D. & Wonderlin, W.F., "Translocon Pores in the Endoplasmic Reticulum Are Permeable to a Neutral, Polar Molecule" (2001) 276 Journal of Biological Chemistry 22655.

High, S. & Dobberstein, B., "The signal anchor sequence interacts with the methionine-rich domain of the 54-kD protein of signal recognition particle" (1991) 113 Journal of Cell Biology 229.

High, S., Anderson, S.S.L., Gorlich, D., Hartmann, E., Prehn, S., Rapoport, T.A., Dobberstein, B., "Sec61p is Adjacent to Nascent Type I and Type II Signal-Anchor Proteins during Their Membrane Insertion" (1993a) 121 Journal of Cell Biology 743.

High, S., Martoglio, B., Gorlich, D., Andersen, S.S.L., Ashford, A.J., Giner, A., Hartmann, E., Prehn, S., Rapoport, T.A., Dobberstein, B., Brunner, J. "Site-specific Photocross-linking Reveals That Sec61p and TRAM Contact Different Regions of a Membrane-inserted Signal Sequence" (1993b) 268 Journal of Biological Chemistry 26745.

Honsho, M., Mitoma, J., Ito, A., "Retention of Cytochrome b_5 in the Endoplasmic Reticulum Is Transmembrane and Lumenal Domain-dependent" (1998) 273 Journal of Biological Chemistry 20860.

Horie, C., Suzuki, H., Sakaguchi, M., Mihara, K., "Characterization of Signal That Directs C-Tail-anchored Proteins to Mammalian Mitochondrial Outer Membrane. Molecular Biology of the Cell" (2002) 13 Molecular Biology of the Cell 1615.

Isenmann, S., Khew-Goodall, Y., Gamble, J., Vadas, M., Wattenberg, B.W., "A Splice-Isoform of Vesicle-associated Membrane Protein-1 (VAMP-1) Contains a Mitochondrial Targeting Signal" (1998) 9 Molecular Biology of the Cell 1649.

Janiak, F., Leber, B., Andrews, D.W., "Assembly of Bcl-2 into Microsomal and Outer Mitochondrial Membranes" (1994) 269 Journal of Biological Chemistry 9842.

Jungnickel, B. & Rapoport, T.A., "A Posttargeting Signal Sequence Recognition Event in the Endoplasmic Reticulum Membrane" (1995) 82 Cell 261.

Kabani, M., Beckerich, J.M., Gaillardin, C., "Sls1p Stimulates Sec63p-Mediated Activation of Kar2p in a Conformation-Dependent Manner in the Yeast Endoplasmic Reticulum" (2000) 20 Molecular and Cellular Biology 6923.

Kalies, KU., Gorlich, D., Rapoport, T.A., "Binding of Ribosomes to the Rough Endoplasmic Reticulum Mediated by the Sec61p-Complex" (1994) 126 Journal of Cell Biology 925.

Kalies, KU., Rapoport, T.A., Hartmann, E., "The β Subunit of the Sec61 Complex Facilitates Cotranslational Protein Transport and Interacts with the Signal Peptidase during Translocation" (1998) 141 Journal of Cell Biology 887.

Kim, P.K., Janiak-Spens, F., Trimble, W.S., Leber, B., Andrews, D.W., "Evidence for Multiple Mechanisms for Membrane Binding and Integration via Carboxyl-Terminal Insertion Sequences" (1997) 36 Biochemistry 8873.

Kim, P.K., Hollerbach, C., Trimble, W.S., Leber, B., Andrews, D.W., "Identification of the Endoplasmic Reticulum Targeting Signal in Vesicle-associated Membrane Proteins" (1999) 274 Journal of Biological Chemistry 36876.

Krieg, U.C., Walter, P., Johnson, A.E., "Photocrosslinking of the signal sequence of nascent preprolactin to the 54-kilodalton polypeptide of the signal recognition particle" (1986) 83 PNAS 8604.

Kuroda, R., Ikenoue, T., Honsho, M., Tsujimoto, S., Mitoma, J., Ito, A., "Charged Amino Acids at the Carboxyl-Terminal Portions Determine the Intracellular Locations of Two Isoforms of Cytochrome b_5" (1998) 273 Journal of Biological Chemistry 31097.

Kurzchalia, T.V., Wiedmann, M., Girshovich, A.S., Bochkareva, E.S., Bielka, H., Rapoport, T.A., "The signal sequence of nascent preprolactin interacts with the 54K polypeptide of the signal recognition particle" (1986) 320 Nature 634.

Kutay, U., Ahnert-Hilger, G., Hartmann, E., Wiedenmann, B., Rapoport, T.A., "Transport route for synaptobrevin via a novel pathway of insertion into the endoplasmic reticulum membrane" (1995) 14 EMBO 217.

Laird, V. & High, S., "Discrete Cross-linking Products Identified during Membrane Protein Biosynthesis" (1997) 272 Journal of Biological Chemistry 1983.

Lan, L., Isenmann, S., Wattenberg, B.W., "Targeting and insertion of C-terminally anchored proteins to the mitochondrial outer membrane is specific and saturable but does not strictly require ATP or molecular chaperones" (2000) 349 Biochemical Journal 611.

Lauring, B., Kreibich, G., Wiedmann, M., "The intrinsic ability of ribosomes to bind to endoplasmic reticulum membranes is regulated by signal recognition particle and nascent-polypeptide-associated complex" (1995a) 92 PNAS 9435.

Lauring, B., Sakai, H., Kreibich, G., Wiedmann, M., "Nascent polypeptide-associated complex protein prevents mistargeting of nascent chains to the endoplasmic reticulum" (1995b) 92 PNAS 5411.

Legate, K., Falcone, D., Andrews, D.W., "Nucleotide-dependent Binding of the GTPase Domain of the Signal Recognition Particle Receptor β-Subunit to the α-Subunit" (2000) 275 Journal of Biological Chemistry 27439.

Liao, S., Lin, J., Do, H., Johnson, A.E., "Both Lumenal and Cytosolic Gating of the Aqueous ER Translocon Pore Are Regulated from Inside the Ribosome during Membrane Protein Integration" (1997) 90 Cell 31.

Linstedt, A.D., Foguet, M., Renz, M., Seelig, H.P., Glick, B.S., Hauri, HP., "A C-terminally-anchored Golgi protein is inserted into the endoplasmic reticulum and then transported to the Golgi apparatus" (1995) 92 PNAS 5102.

Lyman, S., & Schekman, R., "Interaction between BiP and Sec63p Is Required for the Completion of Protein Translocation into the ER of *Saccharomyces cerevisiae*" (1995) 131 Journal of Cell Biology 1163.

Martoglio, B., Hofmann, M.W., Brunner, J., Dobberstein, B., "The Protein-Conducting Channel in the Membrane of the Endoplasmic Reticulum Is Open Laterally toward the Lipid Bilayer" (1995) 81 Cell 207.

Matlack, K.E.S., Misselwitz, B., Plath, K., Rapoport, T.A., "BiP Acts as a Molecular Ratchet during Posttranslational Transport of Prepro-α Factor across the ER Membrane" (1999) 97 Cell 553.

Matlack, K.E.S. & Walter, P., "The 70 Carboxyl-terminal Amino Acids of Nascent Secretory Proteins Are Protected from Proteolysis by the Ribosome and the Protein Translocation Apparatus of the Endoplasmic Reticulum Membrane" (1995) 270 Journal of Biological Chemistry 6170.

McClellan, A.J., Endres, J.B., Vogel, J.P., Palazzi, D., Rose, M.D., Brodsky, J.L., "Specific Molecular Chaperone Interactions and an ATP-dependent Conformational Change Are Required during Post-translational Protein Translocation into the Yeast ER" (1998) 9 Molecular and Cellular Biology 3533.

McClellan, A.J. & Brodsky, J.L., "Mutation of the ATP-Binding Pocket of *SSA1* Indicates That a Functional Interaction Between Ssa1p and Ydj1p Is Required for Post-translational Translocation Into the Yeast Endoplasmic Reticulum" (2000) 156 Genetics 501.

Meisinger, C., Sommer, T., Pfanner, N., "Purification of *Saccharomyces cerevisiae* mitochondria devoid of microsomal and cytosolic contaminations" (2000) 287 Analytical Biochemistry 339.

Meyer, HA., Grau, H., Kraft, R., Kostka, S., Prehn, S., Kalies, KU., Hartmann, E., "Mammalian Sec61 Is Associated with Sec62 and Sec63" (2000) 275 Journal of Biological Chemistry 14550.

Miller, J.D., Willhelm, H., Gierasch, L., Gilmore, R., Walter, P., "GTP binding and hydrolysis by the signal recognition particle during initiation of protein translocation" (1993) 366 Nature 351.

Miller, J.D., Tajima, S., Lauffer, L., Walter, P., "The β Subunit of the Signal Recognition Particle Receptor Is a Transmembrane GTPase that Anchors the α Subunit, a Peripheral Membrane GTPase, to the Endoplasmic Reticulum Membrane" (1995) 128 Journal of Cell Biology 273.

Millman, J. & Andrews, D.W., "Switching the Model: A Concerted Mechanism for GTPases in Protein Targeting" (1997) 89 Cell 673.

Misselwitz, B., Staeck, O., Matlack, K.E.S., Rapoport. T.A., "Interaction of BiP with the J-domain of the Sec63p Component of the Endoplasmic Reticulum Protein Translocation Complex" (1999) 274 Journal of Biological Chemistry 20110.

Mitoma., J. & Ito, A., "The carboxy-terminal 10 amino acid residues of cytochrome b_5 are necessary for its targeting to the endoplasmic reticulum" (1992) 11 EMBO 4197.

Moller, I., Beatrix, B., Kreibich, G., Sakai, H., Lauring, B., Wiedmann, M., "Unregulated exposure of the ribosomal M-site caused by NAC depletion results in delivery of non-secretory polypeptides to the Sec61 complex" (1998a) 441 FEBS Letters 1.

Moller, I., Jung, M., Beatrix, B., Levy, R., Kreibich, G., Zimmermann, R., Wiedmann, M., Lauring, B., "A general mechanism for regulation of access to the translocon: Competition for a membrane attachment site on ribosomes" (1998b) 95 PNAS 13425.

Mothes, W., Prehn, S., Rapoport, T.A., "Systematic probing of the environment of a translocating secretory protein during translocation through the ER membrane" (1994) 13 EMBO 3973.

Motz, C., Martin, H., Krimmer, T., Rassow, J., "Bcl-2 and Porin Follow Different Pathways of TOM-dependent Insertion into the Mitochondrial Outer Membrane" (2002) 323 Journal of Molecular Biology 729.

Mullen, R.T., Lisenbee, C.S., Miernyk, J.A., Trelease, R.N., "Peroxisomal Membrane Ascorbate Peroxidase Is Sorted to a Membranous Network That Resembles a Subdomain of the Endoplasmic Reticulum" (1999) 11 The Plant Cell 2167.

Mullen, R.T. & Trelease, R.N., "The Sorting Signals for Peroxisomal Membrane-bound Ascorbate Peroxidase Are within Its C-terminal Tail" (2000) 275 Journal of Biological Chemistry 16337.

Murphy III, E.C., Zheng, T., Nicchitta, C.V., "Identification of a Novel Stage of Ribosome/Nascent Chain Association with the Endoplasmic Reticulum" (1997) 136 Journal of Cell Biology 1213.

Musch, A., Wiedmann, M., Rapoport, T.A., "Yeast Sec Proteins Interact with Polypeptides Traversing the Endoplasmic Reticulum Membrane" (1992) 69 Cell 343.

Meyer HA., Grau H., Kraft R., Kostka S., Prehn S., Kalies KU., Hartmann E., "Mammalian Sec61 Is Associated with Sec62 and Sec63" (2000) 275 Journal of Biological Chemistry 14550.

Neuhof, A., Rolls, M.M., Jungnickel, B., Kalies, KU., Rapoport, T.A., "Binding of Signal Recognition Particle Gives Ribosome/Nascent Chain Complexes a Competitive Advantage in Endoplasmic Reticulum Membrane Interaction" (1998) 9 Molecular Biology of the Cell 103.

Nicchitta, C.V. & Blobel, G., "Lumenal proteins of the mammalian ER are required to complete protein translocation" (1993) 73 Cell 989.

Nicchitta, C.V., Murphy III, E.C., Haynes, R., Shelness, G.S., "Stage- and Ribosome-Specific Alterations in Nascent-Chain-Sec61p Interactions Accompany Translocation across the ER membrane" (1995) 129 Journal of Cell Biology 957.

Ng, D.T.W., Brown, J.D., Walter, P., "Signal Sequences Specify the Targeting Route to the Endoplasmic Reticulum Membrane" (1996) 134 Journal of Cell Biology 269.

Ng, D.T.W. & Walter, P., "ER Membrane Protein Complex Required for Nuclear Fusion" (1996) 132 Journal of Cell Biology 499.

Ogg, S., Poritz, M., Walter, P., "Signal Recognition Particle Receptor is Important for Cell Growth and Protein Secretion in *Saccharomyces cerevisiae*" (1992) 3 Molecular Biology of the Cell 895.

Ogg, S. & Walter, P., "SRP Samples Nascent Chains for the Presence of Signal Sequences by Interacting with Ribosomes at a Discrete Step during Translation Elongation" (1995) 81 Cell 1075.

Ogg, S., Barz, W.P., Walter, P. (1998) A Functional GTPase Domain, but not its Transmembrane Domain, is Required for Function of the SRP Receptor β-subunit. The Journal of Cell Biology 142, 341-354.

Oliver, J., Jungnickel, B., Gorlich, D., Rapoport, T.A., High, S., "The Sec61 complex is essential for the insertion of proteins into the membrane of the endoplasmic reticulum" (1995) 362 FEBS Letters 126.

Panzner, S., Dreier, L., Hartmann, E., Kostka, S., Rapoport, T.A., "Posttranslational Protein Transport in Yeast Reconstituted with a Purified Complex of Sec Proteins and Kar2p" (1995) 81 Cell 561.

Pilon, M., Schekman, R., Romisch, K., "Sec61p mediates export of a misfolded secretory protein from the endoplasmic reticulum to the cytosol for degradation" (1997) 16 EMBO 4540.

Pilon, M., Romisch, K., Quach, D., Schekman, R., "Sec61p Serves Multiple Roles in Secretory Precursor Binding and Translocation into the Endoplasmic Reticulum Membrane" (1998) 9 Molecular Biology of the Cell 3455.

Plath, K., Mothes, W., Wilkinson, B.M., Stirling, C.J., Rapoport, T.A., "Signal Sequence Recognition in Posttranslational Protein Transport across the Yeast ER Membrane" (1998) 94 Cell 795.

Plath, K. & Rapoport, T.A., "Spontaneous Release of Cytosolic Proteins from Post-translational Substrates before their Transport into the Endoplasmic Reticulum" (2000) 151 Journal of Cell Biology 167.

Pool, M.R., Stumm, J., Fulga, T.A., Sinning, I., Dobberstein, B., "Distinct Modes of Signal Recognition Particle Interaction with the Ribosome" (2002) 297 Science 1345.

Potter, M.D. & Nicchitta, C.V., "Regulation of Ribosome Detachment from the Mammalian Endoplasmic Reticulum Membrane" (2000) 275 Journal of Biological Chemistry 33828.

Potter, M.D. & Nicchitta, C.V., "Ribosome-independent Regulation of Translocon Composition and Sec61α Conformation" (2000) 275 Journal of Biological Chemistry 2037.

Potter, M.D. & Nicchitta, C.V., "Endoplasmic Reticulum-bound Ribosomes Reside in Stable Association with the Translocon following Termination of Protein Synthesis" (2002) 277 Journal of Biological Chemistry 23314.

Prinz, A., Behrens, C., Rapoport T.A., Hartmann E., Kalies, KU., "Evolutionarily conserved binding of ribosomes to the translocation channel via the large ribosomal RNA" (2000a) 19 EMBO 1900.

Prinz, A., Hartmann, E., Kalies, KU., "Sec61p Is the Main Ribosome Receptor in the Endoplasmic Reticulum of *Saccharomyces cerevisiae*" (2000b) 381 Biological Chemistry 1025.

Prinz, W.A., Grzyb, L., Veenhuis, M., Kahana, J.A., Silver, P.A., Rapoport, T.A., "Mutants Affecting the Structure of the Cortical Endoplasmic Reticulum in *Saccharomyces cerevisiae*" (2000c) 150 Journal of Cell Biology 461.

Raden, D. & Gilmore, R., "Signal Recognition Particle-dependent Targeting of Ribosomes to the Rough Endoplasmic Reticulum in the Absence and Presence of the Nascent Polypeptide-associated Complex" (1998) 8 Molecular Biology of the Cell 117.

Raden, D., Song, W., Gilmore, R., "Role of the Cytoplasmic Segments of Sec61α in Ribosome-binding and Translocation-promoting Activities of the Sec61 Complex" (2000) 150 Journal of Cell Biology 53.

Rapiejko, P.J. & Gilmore, R., "Protein Translocation Across the ER Requires a Functional GTP Binding Site in the α Subunit of the Signal Recognition Particle Receptor" (1992) 117 Journal of Cell Biology 493.

Rapiejko, P.J. & Gilmore, R., "Empty Site Forms of the SRP54 and SRα GTPases Mediate Targeting of Ribosome-Nascent Chain Complexes to the Endoplasmic Reticulum" (1997) 89 Cell 703.

Rayner, J.C. & Pelham, H.R.B., "Transmembrane domain-dependent sorting of proteins to the ER and plasma membrane in yeast" (1997) 16 EMBO 1832.

Sadler, I., Chiang, A., Kurihara, T., Rothblatt, J., Way, J., Silver, P., "A Yeast Gene Important for Protein Assembly into The Endoplasmic Reticulum and the Nucleus Has Homology to DnaJ, an *Escherichia coli* Heat Shock Protein" (1989) 109 Journal of Cell Biology 2665.

Sanders, S.L., Whitfield, K.M., Vogel, J.P., Rose, M.D., Schekman, R.W., "Sec61p and BiP Directly Facilitate Polypeptide Translocation into the ER" (1992) 69 Cell 353.

Siegel, V. & Walter, P., "Each of the Activities of the Signal Recognition Particle (SRP) Is Contained within a Distinct Domain: Analysis of Biochemical Mutants of SRP" (1988a) 52 Cell 39.

Siegel, V. & Walter, P., "The affinity of signal recognition particle for presecretory proteins is dependent on nascent chain length" (1988b) 7 EMBO 1769.

Simon, S.M. & Blobel, G., "A Protein-Conducting Channel in the Endoplasmic Reticulum" (1991) 65 Cell 371.

Song, W., Raden, D., Mandon, E.C., Gilmore, R., "Role of Sec61α in the Regulated Transfer of the Ribosome-Nascent Chain Complex from the Signal Recognition Particle to the Translocation Channel" (2000) 100 Cell 333.

Steel, G.J., Brownsword, J., Stirling, C.J., "Tail-anchored Protein Insertion into Yeast ER Requires a Novel Posttranslational Mechanism Which Is Independent of the SEC Machinery" (2002) 41 Biochemistry 11914.

Stirling, C.J., "Protein targeting to the endoplasmic reticulum in yeast" (1999) 145 Microbiology 991.

Figure 19: Intensity of Western blot bands generated by Sss1p and Sec61p polyclonal antibodies increase linearly with protein concentration

To determine if the Kodak Image Station could be used to accurately quantify the intensity of bands from a Western blot, Wt yeast whole cell lysates (of increasing cell number) were separated by SDS-PAGE, transferred to nitrocellulose and decorated with anti-Actin monoclonal, anti-Mir1p, anti-Sec61p or anti-Sss1p polyclonal antibodies. Primary antibodies were conjugated with donkey anti-mouse HRP (Actin) or donkey anti-rabbit HRP (Mir1p, Sec61p, Sss1p) secondary antibodies and developed using Enhanced ChemiLuminescence (ECL) reagent. The net intensity of Western blot bands corresponding to each protein was determined using Kodak 1D Image software. Raw data was compiled using Microsoft Excel software and the mean intensity was plotted against increasing volume of total protein loaded.