SUPPLEMENTARY DISCUSSION

Membrane targeting dynamics from total soluble mRNA.

Cotranslational targeting to the membrane is proposed to involve an SRP-induced elongation arrest after emergence of the first targeting signal to allow time for attachment to the translocon to occur[17,18,58]. Previously, whole-cell ribosome footprints did not provide evidence for an arrest[9], but this could be explained by our finding that most secretory transcripts are already membrane-bound and thus in a post-targeting stage. However, an arrest should be observed in the pre-targeted transcripts found on the soluble-fraction ribosomes. Instead, the predicted increase in ribosome-protected reads was not observed in soluble polysomes translating secretory proteins (Fig. 1b, c and Extended Data Fig. 1d). Moreover, we observed secretory RNCs that remained soluble for hundreds of residues after SS- or TMD- exposure. Thus, the majority of secretory transcripts continue translation on soluble ribosomes well after exposure of the first targeting signal.

We find that cotranslational translocation is prevalent for both SS and TMD containing proteins and is in kinetic competition with elongation. As a result, proteins who's first targeting signal is near the C-terminus are more likely to terminate prior to membrane attachment, thus requiring posttranslational targeting[59]. Proteins with signals further from the C-terminus only require a pioneer round of targeting, and then remain at the membrane through successive rounds of translation. An important attractive feature of this model is that the mRNA pool that requires SRP is limited to newly synthesized mRNAs that will undergo a pioneer round of translation, in line with the lower concentrations of SRP relative to secretory RNCs.

Comparing SRP binding and membrane enrichment indicates SRP binding is rapid and precedes membrane targeting.

Overall, we find excellent correspondence between SRP-enrichment and membrane association (Fig. 2c). Of note, for membrane protein transcripts encoding their first TMD within 250 residues of the C-terminus, there was strong cotranslational enrichment of SRP on soluble RNCs, suggesting binding occurs shortly after signal exposure by the ribosome. Consistently, these soluble SRP-bound transcripts had increased ribosome-protected reads immediately following TMD exposure (Extended Data Fig. 2c–d, as well as the examples in Extended Data Fig. 5c). However, the membrane enrichment of these transcripts was progressively decreased as the TMD was positioned closer to the terminus (Extended Data Fig. 2b). This indicates that SRP-binding occurs rapidly and precedes membrane attachment, but because elongation continues after SRP binding, these SRP-RNC complexes cannot reach the membrane

prior to translation termination. Consistent with this model, a pharmacological elongation block with CHX increased the membrane enrichment of late TMDs and decreased their soluble SRP-enrichment (Fig. 2c). This indicates that these SRP-RNCs with late targeting signals are competent for translocation but without an elongation arrest, their translation terminates prior to membrane targeting.

Translational dynamics of monosomes and polysomes.

Compared to polysomes, total soluble monosomes yield a similar distribution of protected reads across ORFs (Extended Data Fig. 4b, c). However, as expected, monosomes had higher read density at the start codon and end of transcripts. This reflects the likelihood of conversion of monosomes to polysomes due to initiation and back to monosomes due to termination (Extended Data Fig. 4a). Total soluble monosomes were enriched in secretory proteins compared to polysomes obtained from the same sampling of the soluble fraction (Extended Data Fig. 4d). This is consistent with a pioneer round of membrane attachment that preferentially depletes secretory polysomes from the soluble fraction[16,60] (Extended Data Fig. 4e).

Signals do not recruit SRP from within the exit tunnel.

It has been proposed that the presence of a TMD in the ribosomal tunnel suffices to recruit SRP[23,24,61] (Extended Data Fig. 5d). However, analysis of SRP-bound monosomes and polysomes did not support this model, and instead showed that a subset of secretory proteins could only recruit SRP after translation of a targeting signal (Fig. 3b and Extended Data Fig. 5c). For these RNCs, the increase of SRP-bound reads occurs between 50 and 80 codons after synthesis of the first codon in the SS or TMD (Extended Data Fig. 2f). Thus, in these cases SRP directly recognizes the signal exposed by the nascent chain. In addition, the overall lack of SRP enrichment of TA proteins also argues against a model where TMDs recruit SRP from within the exit tunnel (Fig. 2b). While it is possible that a SS/TMD contained in the ribosome exit tunnel may pre-configure a ribosome-bound SRP for impending recognition, this does not appear to be a mechanism for selecting cognate mRNA in and of itself.

Rapid scanning of all ribosomes by SRP cannot explain pre-recruitment.

Early pre-recruitment is the predominant mode of SRP binding to soluble ribosomes translating secretory mRNAs (Fig. 3c). While not consistent with canonical models, we considered whether the reported affinity of SRP for the ribosome could explain our data[26,62,63]. Here, SRP would bind to every ribosome, and the interaction would be strengthened upon synthesis of a SS or TMD. In this model, the SRP-bound monosomes should generate a distribution of reads identical to overall ribosome abundance,

and should include cytonuclear and secretory proteins. This would be reflected in a lack of SRP enrichment for all RNCs except those exposing a targeting signal. This is the opposite of what we observe, since SRP is strongly and specifically enriched in secretory transcripts even when their SS/TMD have not yet been translated. Thus, our data does not support an SRP scanning model but instead suggests that determinants other than the nascent chain contribute to cotranslational SRP recruitment and specificity.

The in vivo role and mechanism of SRP in ER membrane targeting.

Our analysis provides new insights into how yeast SRP selects its substrates and functions in cotranslational targeting *in vivo*. SRP mediates a pioneer round of cotranslational membrane targeting to establish a membrane-bound pool of secretory transcripts. These mRNAs remain ER-associated through subsequent rounds of translation initiation at the membrane. Most secretory protein transcripts associate with SRP cotranslationally, regardless of whether they have a strict genetic SRP requirement for targeting. In this regard, SRP requirement has parallels with chaperone-dependent protein folding in the cytosol. For example, while GroEL binds many proteins in the cell, only some are obligate substrates while others can employ alternative systems when GroEL function is genetically disrupted[64].

Ribosome profiling provides little evidence that SRP-binding to targeting signals induces an elongation arrest. Instead, multiple lines of evidence suggest that membrane targeting competes with continued elongation. Firstly, soluble polysome Ribo-seq profiles show no evidence of SRP-induced elongation pausing or arrest, even though translation attenuation can be clearly observed for rare codons, and poly-basic or proline sequence stretches[65]. Secondly, we observe soluble SRP-bound cytosolic ribosomes exposing multiple transmembrane domains. It will be interesting to determine whether additional factors maintain RNCs exposing multiple TMDs competent for attaching to the membrane and if they are also subject to triage pathways. Finally, SRP binds efficiently to proteins with a late C-terminal signal, but these fail to target cotranslationally to the membrane due to continued elongation and termination.

As a result of the kinetic competition between elongation and targeting, the balance of cotranslational vs posttranslational membrane targeting is not dictated by the properties of the targeting signal, i.e. SS or TMD, but rather by the topology of the secretory protein. Short secreted proteins and those with a late, C-terminal first TMD are translocated posttranslationally. Of note, studies in mammalian cells have also observed posttranslational targeting to the ER for short secretory proteins,

including pre-prolactin and pre-proinsulin, requiring the mammalian homologs of the posttranslational translocon genes SEC62 and SEC63[66–68].

While our data do not support an SRP-induced elongation arrest in our experiments, we do, however, find that a subset of mRNAs have intrinsic features to slow elongation kinetics upon signal exposure. These include the previously reported rare codon clusters[19] but also polybasic stretches[21] of amino acids within the tunnel and proline-rich sequences[22,57]. These multiple elongation attenuation mechanisms may represent a compromise between the evolutionary constraints of the diverse types of secretory proteins and the need to coordinate translation and membrane targeting without triggering mRNA quality control systems that degrade stalled or arrested RNCs[69,70].

In searching for nascent chain determinants that confer specificity for SRP recruitment *in vivo*, we find that for the majority of secretory RNCs, SRP binds stably from the very start of translation. A direct interaction of SRP with SS or TMDs exposed outside the ribosome is also supported by our data, but our experiments indicate that in the cell, SRP is often pre-recruited to RNCs translating cognate substrates before the first targeting signal is translated. These findings indicate SRP recruitment must rely on other transcript features. By focusing on PMP1 and PMP2, we uncover an unanticipated contribution of mRNA elements to client selection that circumvents the need for direct nascent chain recognition. In the case of PMP1 and PMP2, it is the 3′ UTR that influences SRP recruitment, but it is possible that for other secretory transcripts signals in the 5′ UTR, or even in the coding sequence may promote selectivity for SRP. Our results suggest an important role for non-coding mRNA regions in determining the posttranslational fate of encoded proteins, which may extend beyond SRP recruitment[71]. For instance, while SRP-bound RNCs, in general, only target to the membrane after SS/TMD exposure, the 3′ UTRs of PMP1 and PMP2 also confers membrane enrichment. Perhaps the membrane-targeting function of the PMP1 and PMP2 UTRs, which is also dependent on translation, is distinct from its ability to recruit SRP.

Future studies should establish if SRP itself recognizes specific mRNA sequences, or if recruitment involves interaction between SRP and mRNA-binding proteins selective for secretory transcripts[29]. Since puromycin treatment affects SRP-mRNA interactions, any possible mechanism of pre-recruitment should incorporate the role of the ribosome. Interestingly, the Alu domain of SRP contacts the small and large ribosomal subunit interface close to the GTPase center[23,72] and is thus ideally positioned to contact the mRNA, mRNA binding proteins or other ribosome binding factors. It is tempting to speculate that the SRP Alu domain, previously suggested to mediate elongation arrest, participates in the pre-recruitment mechanisms. A functional dissection of the extensive contact of SRP

across the ribosome surface[23,72] may provide a structural basis for understanding the pre-recruitment observed here.

We find that previously defined principles of SRP recruitment, receptor engagement, and handoff to the translocon[73,74] contribute to the function of SRP in eukaryotes, but the greater size and complexity of the appear to lead to a more intricate and spatially regulated SRP and membrane targeting cycle. Recent cryo-EM structures of SRP-RNC complexes in the pre- and post- exposure of a TMD (i.e. scanning and engaged states respectively) show SRP poised to contact the TMD before it emerges from the ribosome[23]. For other secretory proteins, SRP recruitment to the ribosome is initiated by binding a fully exposed SS or TMD in the nascent chain, although this alternate mechanism for selection is exclusively used by a smaller fraction of clients with more hydrophobic targeting signals.

For most secretory mRNAs, SRP binds before targeting signals are synthesized, perhaps recruiting newly exported mRNAs during the pioneer round of translation (Fig. 4e). Pre-recruited SRP may reside on cytosolic RNCs throughout the length of translation. Only upon emergence of a targeting signal from the ribosome, SRP recognizes it and facilitates translocation. Once at the membrane, secretory mRNAs remain bound through subsequent rounds of initiation and translocon engagement. These hydrophobic proteins will no longer compete with soluble proteins for cytosolic quality control machinery.

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