Supporting Information

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SI Methods

Fluorescence Experiments.

Setup and analysis. The binding affinities of SRP for RNCs were determined via equilibrium titrations as previously described (1). FRET was measured between 20 nM Cm-labeled RNCs (donor) and varying concentrations of BODIPY FL labeled Ffh (C421) in the presence of increasing amounts of TF. FRET efficiency \( E \) was calculated from Eq. S1, in which \( F_{DA} \) and \( F_D \) are fluorescence intensities of the donor measured in the presence and absence, respectively, of acceptor. Observed \( E \) values were plotted against SRP concentration, and the data were fitted to Eq. S2,

\[
E = 1 - \frac{F_{DA}}{F_D} \quad [\text{S1}]
\]

\[
E = E_{\text{max}} \left\{ \frac{[\text{RNC}] + [\text{SRP}] + K_d - \sqrt{([\text{RNC}] + [\text{SRP}] + K_d)^2 - 4[RNC][SRP]}}{2[RNC]} \right\} \quad [\text{S2}]
\]

where \( E_{\text{max}} \) is the maximum FRET efficiency at saturating SRP concentrations, and \( K_d \) is the equilibrium dissociation constant of SRP for the RNC. The TF–RNC binding affinities in the presence or absence of SRP were determined analogously, except that FRET between Cm-labeled RNC and BODIPY FL labeled TF (C377) was measured and the term [SRP] in Eq. S2 was replaced by [TF].

The stability of the SRP-FtsY early intermediate and the association rate constants for the SRP-FtsY closed complex were determined using FRET between donor (DACK) and acceptor (BODIPY FL) labeled SRP (C153) and FtsY (C345), respectively, as described (2). In these experiments, SRP was loaded with RNCs at concentrations 5- to 100-fold above their respective \( K_d \) for SRP ± TF to ensure 80–99% occupancy of SRP by the cargo.

For the early complex, equilibrium titrations were carried out using 50 nM RNC-bound, donor-labeled SRP and increasing amounts of acceptor-labeled FtsY in the absence of GTP or any GTP analog. The titrations were carried out with 0 μM or 10–20 μM TF. FRET efficiency was calculated as described and plotted as a function of FtsY concentration. The data were fitted to Eq. S3,

\[
E = E_{\text{max}} \times \frac{[\text{FtsY}]}{K_d + [\text{FtsY}]} \quad [\text{S3}]
\]

where \( E_{\text{max}} \) is the FRET value at saturating amounts of FtsY, and \( K_d \) is the equilibrium dissociation constant of the early intermediate.

SRP-FtsY closed complex assembly rates were determined by mixing 50 nM RNC-bound SRP with varying amounts of FtsY in the presence of 100 μM GppNHP, and the change of fluorescence signal was monitored over time. These time courses were carried out with 0 μM or 10–20 μM TF and were fitted to a single exponential equation to obtain the observed rate constant, \( k_{\text{obsd}} \). Values of \( k_{\text{obsd}} \) were plotted against [FtsY] and fitted to Eq. S4, in which \( k_{\text{on}} \) and \( k_{\text{off}} \) are the rate constants for closed complex assembly and disassembly, respectively.

\[
k_{\text{obsd}} = k_{\text{on}}[\text{FtsY}] + k_{\text{off}}. \quad [\text{S4}]
\]

Mathematical analysis. The first set of simulations in Fig. S5 calculates the amount of time for SRP and FtsY to complete the targeting of RNCs with different signal sequences and length in the presence and absence of TF. First, the fraction of RNC bound to SRP \( F \) was determined using Eq. S5,

\[
F = \frac{[\text{SRP}]}{K_d + [\text{SRP}]} \quad [\text{S5}]
\]

in which an estimated cellular SRP concentration of 400 nM was used, and \( K_d \) values for SRP–RNC binding were from Figs. 1 and 5. Next, the observed rate constant for cargo targeting to the membrane, \( k \), was calculated using Eq. S6,

\[
k = k_{\text{on}} \times [\text{FtsY}] \quad [\text{S6}]
\]

where an estimated cellular [FtsY] of 4–10 μM was used, and \( k_{\text{on}} \) values for SRP-FtsY complex assembly were from Figs. 4 and 5. The time needed by SRP and FtsY to complete targeting (\( \tau \)) was determined using Eq. S7,

\[
\tau = 1/(F \times k) \quad [\text{S7}]
\]

The second set of simulations in Fig. 7 A–D was carried out as described (3). Briefly, we calculated the fraction of each cargo (FtsQ/2A8L, EspP, and phoA) that can be successfully targeted by the SRP and SRP receptor within a given time window, \( t_t \), before the nascent chain surpasses the critical length of >140 amino acids. Calculations used the kinetic and thermodynamic parameters determined in the absence or presence of saturating amounts of TF. In the first step, the percentage of RNCs that bind the SRP, \( P(1) \), was calculated from Eq. S8,

\[
P(1) = \frac{[\text{SRP}]}{K_d + [\text{SRP}]} \times 100 \quad [\text{S8}]
\]

using an [SRP] of 400 nM and \( K_d \) values for RNC–SRP binding determined in Fig. 1E. In the second step, the percentage of cargo that remains in the SRP pathway after SRP-FtsY closed complex assembly, \( P(2) \), was calculated from Eq. S9,

\[
P(2) = P(1) \times \left( 1 - e^{-k_{\text{off}}[\text{FtsY}] \times c \times t_t} \right) \quad [\text{S9}]
\]

using an [FtsY] of 2 μM, \( k_{\text{on}} \) values from Fig. 4E, a time window \( t_t \) of 6 s to complete targeting (before the nascent chain reaches 140 amino acids), and a correction factor \( c \) of 10 for the difference of translation elongation rates in vitro versus in vivo. For RNC-FtsQ, \( k_{\text{off}}[\text{FtsY}] \) was replaced by the rate constant of

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rearrangement from the early to the closed complex, $k_{\text{rearrange}} = 0.6 \text{s}^{-1}$, because at high FtsY concentrations, formation of the SRP•FtsY closed complex is rate-limited by this rearrangement (3, 4). In the last step, the percentage of cargo that is successfully transferred to the translocon before GTP is hydrolyzed, $P(3)$, was calculated from Eq. S7,

$$P(3) = P(1) \times P(2) \times e^{-k_{\text{GTPase}} \times t_{\text{transfer}}}$$

using the GTP hydrolysis rate constants, $k_{\text{GTPase}}$, obtained in Zhang et al. (4, 5). The $t_{\text{transfer}}$ represents the time window for cargo unloading and was estimated to be $\sim 3 \text{s}$ previously (3).


Fig. S1. The SRP targeting pathway. In step 1, SRP recognizes signal sequences and binds to the RNC. In step 2, SRP associates with its receptor to form the transient early complex. In step 3, the early intermediate rearranges to form the stable closed complex, which also allows the SRP•RNC complex to be localized to the SecYEG translocon on the target membrane. In step 4, cargo is handed over to the translocation machinery. In step 5, SRP and FtsY disassemble upon GTP hydrolysis. Highlighted in red are three distinct checkpoints that allow SRP to selectively reject incorrect cargo from the pathway (3, 6). These include cargo binding, induced SRP-FtsY assembly, and kinetic proofreading through GTP hydrolysis.
**Fig. S2.** Experimental design to distinguish different models for how TF affects SRP binding to RNC, related to Fig. 1. (A and B) Depiction of models in which SRP and TF binding to RNC are strictly competitive (A) or anticooperative (B) with one another (Left), and simulation of the effect of TF on RNC-SRP binding curves predicted by each model (Right). Simulations used a \( K_d \) value for RNC–SRP binding of 2 nM, a \( K_d \) value for RNC–TF binding of 0.5 \( \mu \)M, and for the model in B, a 20-fold weakening effect of TF on SRP–RNC binding (\( K_d^{SRP,T} = 20 K_d^{SRP} \)). (C) Representative equilibrium titrations for RNC\(_{3A7L}\)–SRP binding in the presence of increasing TF (indicated as increasing shades of red). The data were fitted to Eq. S2 and yielded the following parameters: apparent \( K_d \) values of 2.8 nM, 6.0 nM, 12.1 nM, 23.4 nM, 36.4 nM, and 39.3 nM and FRET end points of 0.80, 0.72, 0.65, 0.55, 0.45, and 0.37, respectively, with 0, 1 \( \mu \)M, 4 \( \mu \)M, 10 \( \mu \)M, 20 \( \mu \)M, and 50 \( \mu \)M TF present. (D) Equilibrium titrations for RNC\(_{EspP}\)–SRP binding in the presence increasing TF (indicated as increasing shades of red). The data were fitted to Eq. S2 and gave apparent \( K_d \) values of 15.8 nM, 17.2 nM, 21.5 nM, and 21.7 nM and FRET end points of 0.36, 0.29, 0.12, and 0.02, respectively, with 0 \( \mu \)M, 0.1 \( \mu \)M, 0.3 \( \mu \)M, and 1 \( \mu \)M TF present. Error bars are S.D from two to three measurements. (E) Summary of the effect of TF on the apparent RNC–SRP binding affinity for the different substrates. Error bars are SD from two to three measurements or error estimates from fit of data, whichever is greater. (F) Simulated effect of TF on the apparent RNC–SRP binding affinity, as predicted by models in which SRP and TF binding are strictly competitive (red), anticooperative (purple), or independent (green) of one another. The data for the competitive and anticooperative models are from the simulation results in Fig. S2 A and B.
Fig. S3. SRP weakens the binding of TF to different RNCs, related to Fig. 2. (A) Representative equilibrium titrations for the binding of RNC \textit{3A7L} to wild-type TF (●) or to the FRK→AAA mutant (○). The data were fitted to Eq. S2 and gave $K_d$ values of 2.73 nM and 8.50 nM for wild-type and mutant TF, respectively. (B and C) Representative equilibrium titrations for RNC-TF binding in the presence of increasing SRP concentration (indicated as increasing shades of red). The data were fitted to Eq. S2 and yielded the following parameters: (B) apparent $K_d$ values for RNC\textit{3A7L} binding of 2.7 nM, 9.0 nM, 19.5 nM, 27 nM, and 30 nM and FRET end points of 0.34, 0.34, 0.40, 0.42, and 0.52, respectively, with 0 nM, 100 nM, 200 nM, 400 nM, and 800 nM SRP present and (C) apparent $K_d$ values for RNC\textit{EspP} binding of 6.2 nM, 10.5 nM, 14.7 nM, and 21 nM and FRET end points of 0.54, 0.43, 0.40, and 0.47, respectively, with 0 nM, 200 nM, 400 nM, and 800 nM SRP present. (D) Summary of the effect of SRP on the apparent RNC-TF binding affinity for the different substrates. Error bars are SD from two to three measurements or error estimates from fit of data (for \textit{3A7L}), whichever is greater.

Fig. S4. Effect of TF on the apparent binding affinities of SRP for RNCs with longer nascent chains, related to Fig. 5. (A–C) Equilibrium titrations for SRP binding to RNC\textit{FtsQ-135} (A), RNC\textit{3A7L-130} (B), and RNC\textit{phoA-130} (C) in the presence of increasing amounts of TF, as denoted by increasing shades of red. The data were fitted to Eq. S2 and yielded the following parameters: apparent $K_d$ values of 7.7 nM, 6.4 nM, 9.7 nM, 13.0 nM, 39.7 nM, and 38.5 nM and FRET end points of 0.41, 0.30, 0.29, 0.18, and 0.19 with 0, 0.5 μM, 1 μM, 5 μM, 10 μM, and 30 μM TF, respectively, for RNC\textit{FtsQ-135}; apparent $K_d$ values of 3.8 nM, 5.2 nM, 14.9 nM, 25.4 nM, 42.7 nM, 51.8 nM, and 53.2 nM and FRET endpoints of 0.58, 0.41, 0.38, 0.36, 0.32, 0.17 and 0.15 with 0 μM, 0.2 μM, 0.5 μM, 1 μM, 2 μM, 10 μM, and 30 μM TF, respectively, for RNC\textit{3A7L-130}; and apparent $K_d$ values of 3.8 nM, 5.2 nM, 14.9 nM, 25.4 nM, 42.7 nM, 51.8 nM, and 53.2 nM and FRET endpoints of 0.44, 0.46, 0.32, 0.22, 0.14, 0.10, and 0.09 with 0 μM, 0.1 μM, 0.2 μM, 0.5 μM, 1 μM, 2 μM, 5 μM, and 10 μM TF, respectively, for RNC\textit{phoA-130}. Error bars are SDs from 2 to 3 experiments. Some error bars may not be visible.
Fig. S5. Mathematical analysis simulating the time needed by SRP and FtsY to complete targeting of RNCs with different signal sequences and nascent chain lengths. (A) Fraction of RNC bound to SRP. (B and D) The observed membrane targeting rates, determined using the closed complex assembly rate constants and estimated cellular FtsY concentrations of 4 μM (B) and 10 μM (D). (C and E) The amount of time needed by SRP and FtsY to target various RNCs in the absence (light gray and pink) and presence (dark gray and dark pink) of TF, calculated from the parameters obtained in A and in B and D. The red dashed line denotes the time to complete translation for a bacterial protein of average length (330 ± 5 amino acids) with an elongation rate of 20 amino acids per second (7, 8).