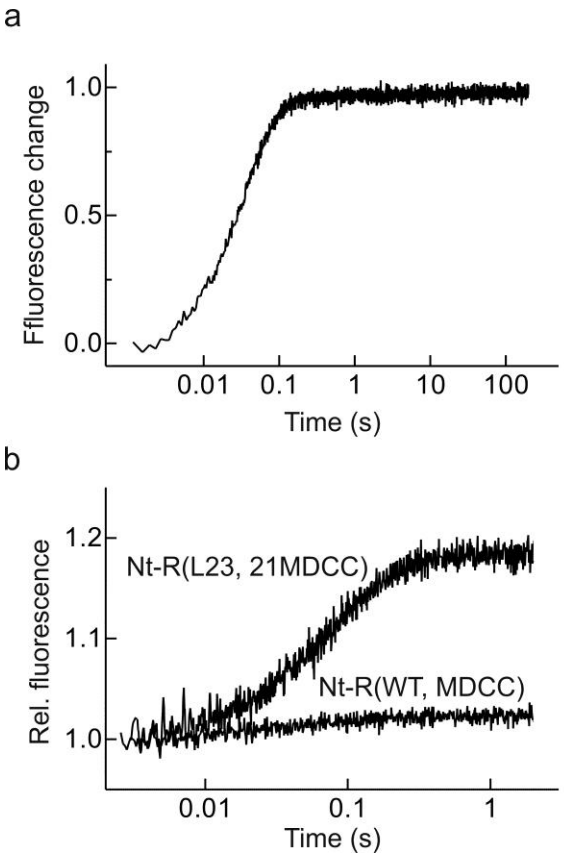
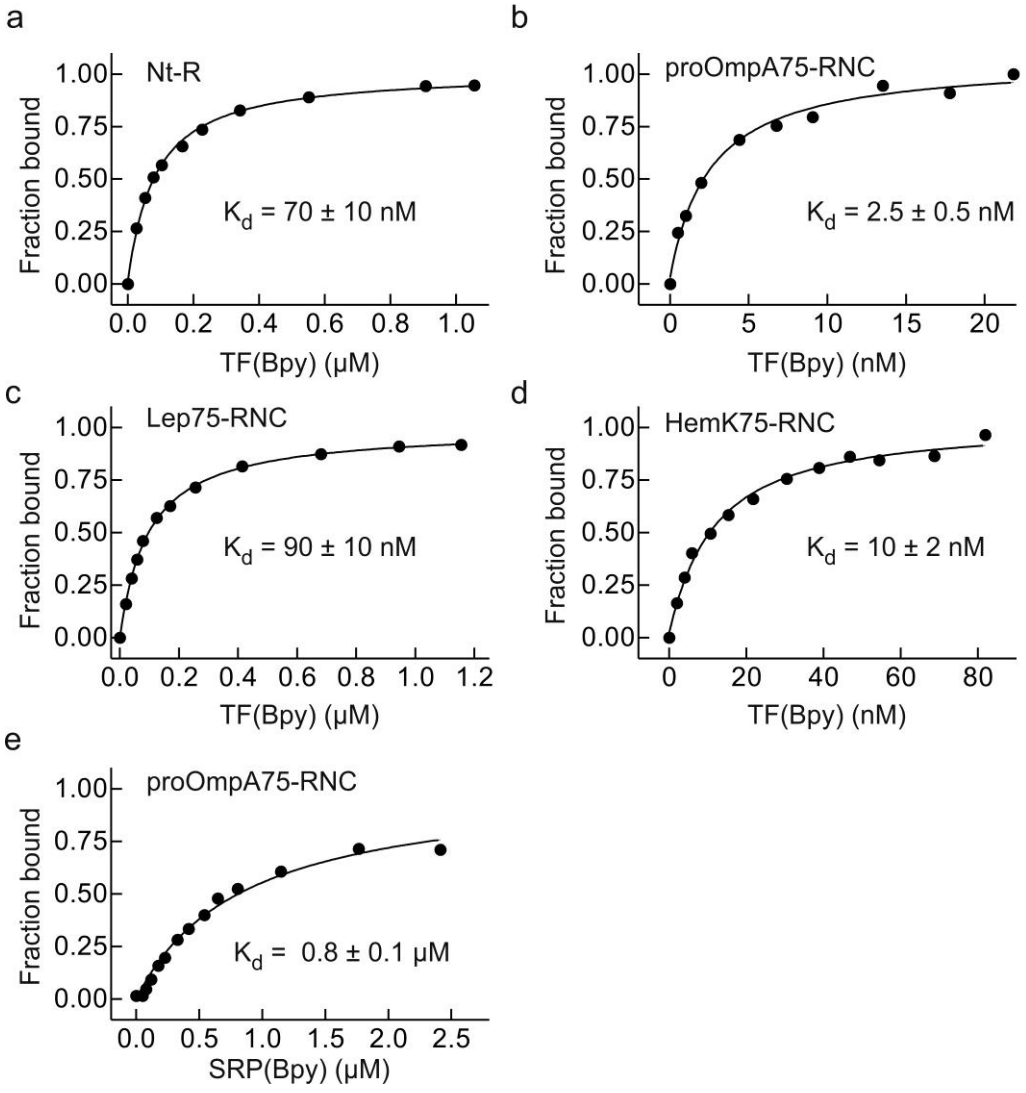
Supplementary Figures

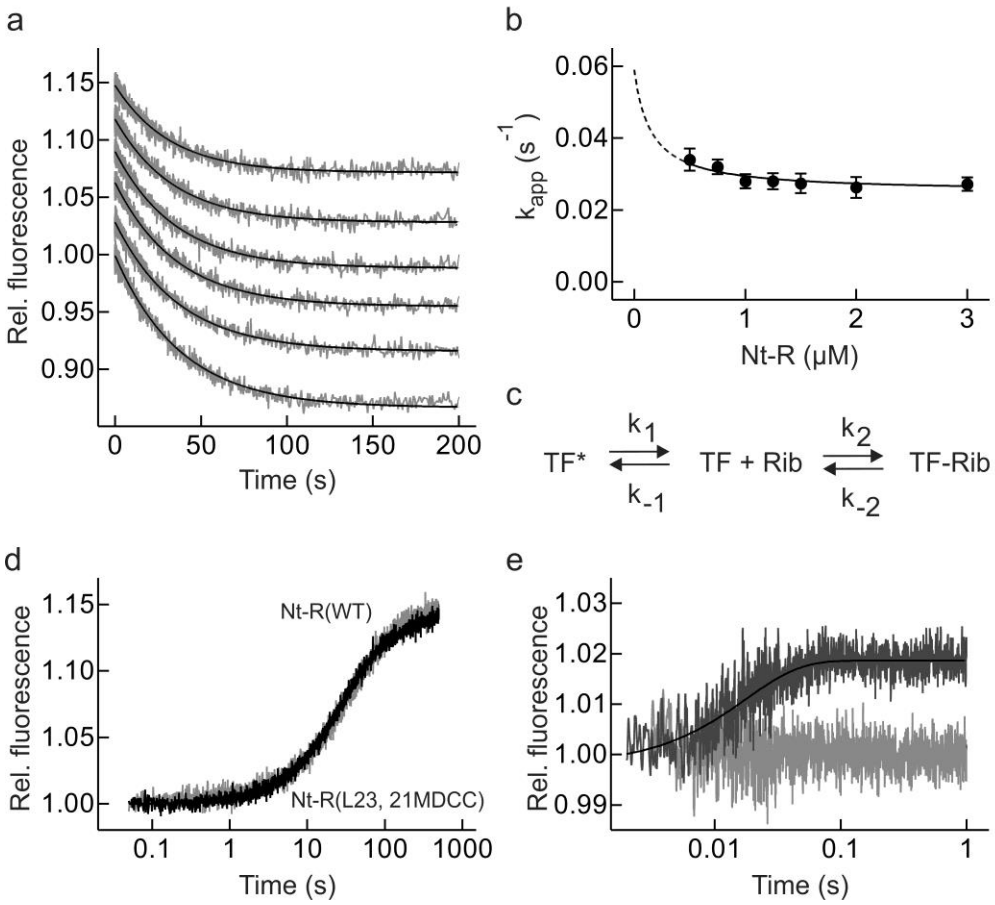


Supplementary Figure 1. Kinetics of TF(Bpy) binding to ribosomes. (**a**) TF(Bpy) binding to MDCC-labeled ribosomes (1 μM) (Materials and Methods). (**b**) Comparison of TF(Bpy) binding to ribosomes containing MDCC-labeled or unmodified L23. TF(Bpy) (0.6 μM) was rapidly mixed with ribosomes (50 nM) containing wild-type L23 (lower trace) or L23(S21C) (upper trace) that had been reacted with MDCC, and Bpy fluorescence was monitored over time.



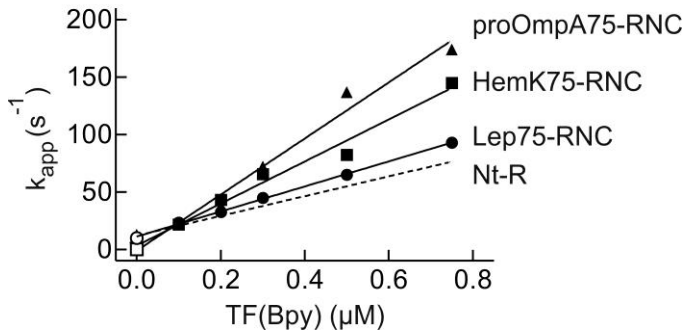
Supplementary Figure 2. Equilibrium titrations of TF and SRP binding to ribosomes and RNCs. (**a**) TF(Bpy) binding to non-translating ribosomes (Nt-R). (**b**) TF(Bpy) binding to proOmpA75-RNC. (**c**) TF(Bpy) binding to Lep75-RNC. (**d**) TF(Bpy) binding to HemK75-RNC. (e) SRP(Bpy) binding to proOmpA75-RNC. Complex formation was monitored by FRET between MDCC-labeled ribosomes or RNCs and Bpy-labeled TF or SRP, measuring donor (MDCC) fluorescence. Concentrations of ribosomes or RNCs were 2.5 nM. Binding curves were

evaluated by non-linear fitting using a quadratic equation for ligand binding to one site (Materials and Methods).

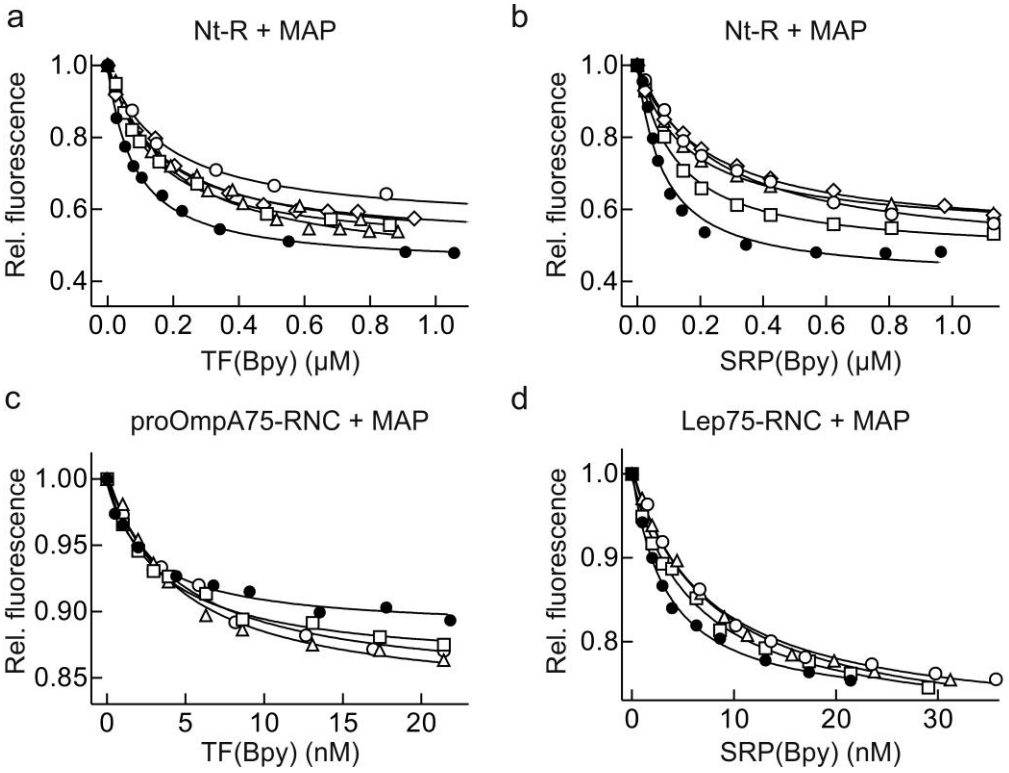


Supplementary Figure 3. Kinetics of the interaction of TF(BADAN) with ribosomes. (**a**) Time courses of TF(BADAN) binding to vacant ribosomes. TF(BADAN) (0.25 μM) was rapidly mixed with ribosomes (0.5 to 3 μM, bottom to top), and BADAN fluorescence (excitation at 390 nm) was measured after passing a cut-off filter (KV500, Schott). For clarity, the traces for ribosome concentrations higher than 0.5 μM are off-set by 0.025 units each on the Y-axis. Single-exponential fits yielded the k_{app} values plotted in panel (b). (**b**) Concentration dependence. The solid line is calculated from the rate constants resulting from the global fits in (a) for the model depicted in (c). The dotted line is predicted by the model in panel (c); measurements at concentrations lower than 0.5 μM were not feasible due to loss of signal. (**c**) Two-step model of TF(BADAN) binding to ribosomes. The binding traces were evaluated by

global fitting (Micromath, Scientist) on the basis of a two-step model, depicted in panel (c). The model assumes that a slow internal TF equilibrium, which leads to the observed change of BADAN fluorescence, precedes rapid ribosome binding, which itself is not reported by BADAN. Rate constants of binding and dissociation, k_2 = 85 $\mu M^{-1}s^{-1}$ and k_{-2} = 12 s^{-1}, were taken from Figure 2 and included in the global fit as fixed values. The best fits (smooth lines in panel (a)) were obtained with k_1 = 0.025 ± 0.001 s^{-1} and k_{-1} = 0.034 ± 0.003 s^{-1}, an 11.4% fluorescence change between TF* and TF, and no fluorescence change upon TF−ribosome complex formation. (**d**) Dissociation. The complexes of TF(BADAN) (0.25 µM) with unlabeled (gray trace) or MDCC-labeled (black trace) ribosomes (1.0 µM) were rapidly mixed with an excess of unlabeled TF (5 µM) and the change of BADAN fluorescence was monitored. (**e**) Dissociation of TF(QSY) from Bpy-labeled Lep35-RNC. The complex of TF labeled with the non-fluorescent FRET acceptor QSY9 at position 99 (0.5 µM) and Lep35-RNC carrying a Bpy label at the N terminus of the nascent chain (0.25 µM) was rapidly mixed with unlabeled TF (5 µM) and Bpy fluorescence was monitored over time (dark-gray trace). Single-exponential fitting (smooth line) yielded a time constant around 50 s^{-1}. In the control experiment, the initial complex was formed with unlabeled TF (light-gray trace).



Supplementary Figure 4. Kinetics of TF(Bpy) binding to RNCs. MDCC-labeled RNCs (25 nM), as indicated, were rapidly mixed with increasing amounts of TF(Bpy), and the change in Bpy acceptor fluorescence excited by FRET via MDCC was monitored. Time courses were fitted with double-exponential (proOmpA-RNC, HemK-RNC) and single-exponential (Lep-RNC) functions to yield k_{app} values that were plotted against the TF(Bpy) concentration. Values of k_{app1} (not shown) were equal to the rates observed with non-translating ribosomes (dotted line, taken from Fig. 2b) or Lep75-RNC; the second step, k_{app2}, represents TF(Bpy) binding to RNCs carrying a nascent chain to which TF binds (proOmpA, HemK) and was not observed with Lep-RNC or non-translating ribosomes. Open symbols represent k_{off} values directly measured by chase (Fig. 2d) which were included in the fits. The kinetic parameters k_{on} and k_{off} derived from the plots by linear fitting are summarized in Table 1.



Supplementary Figure 5. Concurrent binding of MAP and TF or SRP to ribosomes and RNCs. Ribosome-TF(Bpy) complex formation was monitored by the MDCC fluorescence decrease due to FRET. Concentrations of MAP (μM) were the following: None, ●; 1.0, □; 3.0, △; 4.0, ◇; 5.0, ○. To determine K_d values, titration curves were fitted using a quadratic equation for ligand binding to one site. (**a**) Effect of MAP on TF(Bpy) binding to MDCC-labeled non-translating ribosomes. (**b**) Effect of MAP on SRP(Bpy) binding to non-translating ribosomes. SRP(Bpy) binding was monitored by FRET, as in (a). (**c**) Effect of MAP on TF(Bpy) binding to proOmpA-RNC. (**d**) Effect of MAP on SRP(Bpy) binding to Lep-RNC. In the titrations shown in panels (a) to (d), K_d values of TF(Bpy) or SRP(Bpy) binding remained unaffected (within a factor of two) at MAP concentrations up to 5 μM.

Kinetics of the interactions of BADAN-labeled TF with ribosomes

The rapid association-dissociation equilibrium of TF and ribosomes observed here using L23-labeled ribosomes and TF labeled at position 99 differs substantially from the previously reported very low association and dissociation rates of the TF complexes with ribosomes that have been observed with TF labeled with BADAN at position 14 [1-3]. The reported half-life times of those complexes were 10 s or higher, rather than <0.1 s as we observe, and K_d values were reported to be in the µM range, compared to our values of around 0.1 µM. For comparison, we analyzed the kinetics of TF(BADAN)−ribosome complex formation and dissociation at our experimental conditions (Supplementary Fig. 3). We also observed that binding of TF(BADAN) to ribosomes gives rise to a very slow fluorescence decrease ($k_{app} \approx 0.03\ s^{-1}$) and that the dissociation of the TF-ribosome complex is accompanied by a slow fluorescence increase ($k_{app} \approx 0.03\ s^{-1}$), largely consistent with previous data.

The slow conversion presumably does not reflect the dissociation of (non-binding) TF dimers into (binding) monomers, as the reported half-life time of the dimer is rather short (around 1 s) [2]. Furthermore, a model in which initial complex formation of TF with ribosomes is followed by a slow rearrangement can be excluded, as in the chase experiments monitored by FRET no slow step was observed (Fig. 2). According to our kinetic analysis, one possibility to explain the apparent low rates of association and dissociation may be a slow internal TF(BADAN) equilibrium with forward and backward rate constants of around $0.03\ s^{-1}$ that limits the observed rate of the intrinsically rapid ribosome binding step. Such a model would be consistent with the observed concentration-independent k_{app} values (Supplementary Fig. 3b); the model predicts an increase of k_{app} at lower ribosome concentrations (dotted line) which could, however, not be verified experimentally because the signal change was too small at ribosome concentrations below 0.5 µM.

We confirmed that the MDCC fluorophore attached to ribosomal protein L23 had no influence on the association kinetics of BADAN-labeled TF (Supplementary Fig. 3d). An additional control was performed using different labels on the ribosome and on TF for monitoring the dissociation of the complex. As FRET donor we used Bpy at the N terminus of the nascent chain in Lep35-RNC [4] and as (non-fluorescent) FRET acceptor we used QSY9 at position 99 in TF (Supplementary Fig. 3e). This complex also dissociated rapidly (half-life time about 0.015 s), even somewhat more rapidly than the complex with L23-labeled ribosomes.

From these data we conclude that the different kinetics observed in our FRET measurements and by BADAN fluorescence are to be attributed to the BADAN label in TF.

It has been reported that the kinetic stability of TF−ribosome complexes, determined by monitoring the fluorescence of BADAN-labeled TF, did not increase much, up to about three-fold, when TF-specific nascent peptide chains of increasing lengths were presented on the ribosome, whereas the apparent binding rates increased, in some cases up to ten-fold [2,3]. By contrast, our experiments with TF labeled with Bpy at position 99 revealed that the exposure of nascent chains comprising binding sites for TF (proOmpA and HemK), which in our experiments were restricted to a length of 75 amino acids, strongly stabilized the TF−RNC complexes (up to 30-fold slower dissociation), compared to non-translating ribosomes, and had only small effects on the association rate constants (up to three-fold increase) (Table 1).

Supplementary References

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2. Kaiser, C.M. et al. Real-time observation of trigger factor function on translating ribosomes. *Nature* **444**, 455-460 (2006).

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4. Holtkamp, W. et al. Dynamic switch of the signal recognition particle from scanning to targeting. *Nat Struct Mol Biol 19*, 1332-1337 (2012).