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The structural basis of FtsY recruitment and GTPase activation by SRP RNA

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Summary

The universally conserved Signal Recognition Particle (SRP) system mediates the targeting of membrane proteins to the translocon in a multistep process controlled by GTP hydrolysis. Here we present the 2.6Å crystal structure of the GTPase domains of the *E. coli* SRP protein (Ffh) and its receptor (FtsY) in complex with the *tetraloop* and the *distal region* of SRP-RNA, trapped in the activated state. The structure reveals the atomic details of FtsY recruitment and, together with biochemical experiments, pinpoints G83 as the key RNA residue that stimulates GTP hydrolysis. Insertion of G83 into the FtsY active site orients a single glutamate residue provided by Ffh (E277) triggering GTP hydrolysis and complex disassembly at the end of the targeting cycle. The complete conservation of the key residues of the SRP-RNA and the SRP protein implies that the suggested chemical mechanism of GTPase activation is applicable across all kingdoms.

Introduction

The Signal Recognition Particle (SRP) is a universally conserved component of the cellular protein targeting machinery. It binds to ribosome nascent chain complexes (RNCs) that expose a SRP signal sequence and delivers them to the target membrane via interactions with the SRP receptor. The RNC is then transferred to the translocon, which mediates the co-translational transport of the emerging polypeptide across the lipid bilayer and also guides the insertion and folding of membrane proteins (Doudna and Batey, 2004; Egea et al., 2005; Saraogi et al., 2011, Akopian et al., 2013a).

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In bacteria, SRP consists of the ~50 kDa signal recognition particle protein Ffh and a 4.5S SRP RNA. The methionine-rich M-domain of Ffh interacts with SRP RNA and the signal sequence (Zopf et al., 1990; Keenan et al., 1998; Batey et al., 2000). The NG domain has a Ras-like guanosine triphosphatase (GTPase) fold with an SRP-specific insertion (insertion box domain, IBD) and an N-terminal helical bundle that mediates interactions with the ribosome (Freyman et al., 1997; Halic et al., 2004; Schaffitzel et al., 2006) (Figure S1). In *E. coli*, the 100 nucleotide SRP RNA forms an elongated, mostly helical hairpin structure with a GNRA type *tetraloop*. The Ffh M-domain binding element is located close to the *tetraloop*, while the *distal* binding site is located 100 angstrom (Å) away from the *tetraloop*, towards the 5'-3' end of the hairpin (Ataide et al., 2011). The prokaryotic SRP receptor (FtsY) is a single protein consisting of an NG domain homologous to Ffh (Montoya et al., 1997) and an additional acidic domain (A domain) that interacts with the SecYEG translocon (Weiche et al., 2008). Ffh and FtsY belong to the signal recognition, MinD, and BioD (SIMBI) class of GTPases and their GTPase activity is regulated by dimerization (Wittinghofer and Vetter, 2011). In the heterodimer, both polypeptides contribute nearly identical residues to a composite active site that binds two guanosine triphosphate (GTP) nucleotides (Egea et al., 2004; Focia et al., 2004).

The SRP RNA serves as a platform for the association of Ffh and FtsY in the membrane targeting process and is also required for efficient GTPase activation (Peluso et al., 2000; 2001; Zhang et al., 2008). Ffh mediates initial interactions between SRP and the RNC: The M-domain recognizes the emerging signal sequence and the NG domain contacts ribosomal protein L23 on the opposite side of the exit tunnel (Gu et al., 2003; Halic et al., 2004; Schaffitzel et al., 2006). The targeting process begins with the formation of an *early complex* stabilized by an interaction between FtsY and the *tetraloop* (Zhang et al., 2007; Shen and Shan, 2010). Subsequent formation of the *closed complex* involves the GTP-mediated dimerization of FtsY and Ffh via their NG-domains (Egea et al., 2004; Focia et al., 2004; Zhang et al., 2008). The GTP-bound NG-domain heterodimer then undergoes a large-scale repositioning to the *distal region* of the SRP RNA, which is facilitated by a helical linker between the RNA-bound M-domain and the NG domain of Ffh (Ataide et al., 2011). This SRP remodeling is required for efficient GTPase activation, which leads to the dissociation of the complex and handover of the cargo (the ribosome nascent chain complex) to the translocon (Jagath et al., 2001; Siu et al., 2007; Estrozi et al., 2011).

While structural and biochemical evidence shows that both *tetraloop* (Jagath et al., 2001; Egea et al., 2004; Focia et al., 2004; Siu et al., 2007; Estrozi et al., 2011) and *distal* interactions (Ataide et al., 2011; Shen et al., 2012) with the SRP RNA are required for the efficient formation of an Ffh:FtsY complex, GTP hydrolysis and protein translocation, the lack of high resolution models has so far limited our understanding of the SRP RNA in the targeting process. We have determined the 2.6Å crystal structure of two Ffh:FtsY NG domain heterodimers, bound to the *tetraloop* and the *distal region* of SRP RNA in presence of guanosine diphosphate (GDP) and aluminum tetrafluoride (AlF₄), a mimic of the GTP hydrolysis transition state (Wittinghofer, 1997). In combination with the biochemical evidence presented herein, the complex reveals the structural basis for the regulation of the SRP pathway at several stages, including the recruitment of FtsY and the RNA-mediated GTPase-activation upon SRP remodeling.

Results

GDP-aluminum fluoride stabilizes the interaction between the Ffh and FtsY NG heterodimer and SRP RNA

Binding of transition state analogs such as GDP-AlF_x to GTPases increases the affinity for their cognate GTPase activating proteins (GAPs) and therefore promotes the formation of a

hydrolysis competent complex (Gasper et al., 2009). Since it was established that the RNA is directly responsible for GTPase activation in the SRP system (Ataide et al., 2011) we decided to test whether the presence of AlF_x enhances binding of the Ffh and FtsY NG domain heterodimer to SRP RNA. Dimerization of Ffh:FtsY NG-domains in the presence of either of GMPPCP or GDP AlF_x was confirmed by analytical size exclusion chromatography. Then, a constant amount of a shortened SRP RNA construct (described in detail below) was incubated with increasing concentrations (1:1 to 1:10 fold molar ratio) of proteins in the presence of either nucleotide analog. In presence of GDP- AlF_x , but not GMPPCP, the RNA elution volume shifts, indicating the formation of a larger complex (Figure 1, S2). Hence, the affinity of the NG heterodimer for the RNA is increased in presence of the transition state mimic, relative to the ground state analog. Based on the RNA concentration of 5 μM and the dilution effect on the SEC column, we estimate the dissociation constant of the NG-heterodimer:RNA complex in the presence of GDP: AlF_x in the order of 0.5-1 μM .

Overview of the crystal structure

In order to obtain further insights into the mechanism of receptor recruitment and GTPase activation by the SRP RNA we co-crystallized the NG domains of FtsY and Ffh with GDP: AlF_x and shortened SRP RNA (also used in the gel filtration experiments above). To facilitate crystallization, the RNA construct was truncated to the *tetraloop* and the *distal region* identified previously as responsible for the binding of the NG heterodimer and GTPase activation by structural and biochemical experiments (Ataide et al., 2011; Shen et al., 2012) (Figure 2). Based on our current understanding of complexes involved in SRP targeting (Akopian et al., 2013a) omitting the M-domain and the RNA region it interacts with is unlikely to perturb physiological interactions between the SRP RNA and the NG domains of Ffh and FtsY. Crystals diffracted to 2.6Å and the structure was solved by molecular replacement (Table 1). Coordinates and structure factors have been deposited in the Protein Data Bank (accession code 4c7o). The asymmetric unit consists of a single SRP RNA molecule that interacts simultaneously with two Ffh:FtsY NG-domain heterodimers. One is bound to the *tetraloop*, and the second interacts with the *distal region* of the SRP RNA (Ataide et al., 2011). Two GDP- AlF_x complexes are bound in a head-to tail orientation at each NG-domain interface, and a flipped-out RNA base (corresponding to base G83 of full length *E. coli* SRP RNA) projects into the NG-domain interface, pointing toward the FtsY-bound AlF_4 moiety, while the G83 binding site of the *tetraloop*-bound heterodimer is occupied by a free nucleotide from the crystallization buffer.

The structure captures a snapshot of two distinct stages of the SRP cycle: (1) The *closed* GTPase heterodimer that is formed after cargo recognition, just before it detaches from the *tetraloop*, and (2) the *activated state* of GTP hydrolysis that occurs after the heterodimer has re-localized to the *distal region* of the SRP RNA (Ataide et al., 2011; Zheng et al., 2011; Shen et al., 2012) (Figure 2).

The *tetraloop* is recognized by FtsY helix 2 and Insertion Box Domain (IBD) helix B

The interaction between the *tetraloop* and FtsY –although transient in nature (Shen and Shan, 2010; Estrozi et al., 2011)- is crucial for successful complex formation and targeting *in vivo* and *in vitro* (Peluso et al., 2000; Siu et al., 2007). The presence of the SRP RNA accelerates the formation of the GTPase heterodimer (Peluso et al., 2000; 2001; Siu et al., 2007; Zhang et al., 2008; Gasper et al., 2009) via the *tetraloop*. As proposed earlier (Peluso et al., 2000; Zhang et al., 2008) the SRP RNA acts as an initial attachment point that increases the local concentration of FtsY and orients it in the correct orientation to form an *early complex* with Ffh (Zhang et al., 2008; Estrozi et al., 2011). Upon GTP binding, the NG

heterodimer rearranges into the *closed*, tightly associated state observed in the crystal structure (Egea et al., 2004; Focia et al., 2004).

The presented crystal structure reveals the atomic details of the SRP RNA-FtsY interaction at the *tetraloop*, which are in agreement with the contacts observed for the early complex visualized by electron microscopy (Estrozi et al., 2011). The contacts involve three clusters of universally conserved residues (Figure 3, S3); First, a highly basic patch on FtsY helix 2 (Shen and Shan, 2010) forms salt bridges with the *tetraloop* backbone via residues K399, R402 and to a lesser extent K406. Biochemical experiments demonstrated that these residues are critical for complex formation (Egea et al., 2004; Shan et al., 2004; Shen and Shan, 2010) and also play a role in binding to the SRP RNA on the distal site (see below and Figure S6). The second recognition patch forms aromatic interactions with bases two to four of the *tetraloop* and consists of the hydrophobic residues L407 and phenylalanine F365 of helix 2 and IBD helix B, respectively. The hydrogen-bonding pattern between D366 (IBD helix B) and the exocyclic amine of the second base in the *tetraloop* motif is specific for a guanosine in this position. The exocyclic amine of the second *tetraloop* base also hydrogen bonds to the backbone carbonyl oxygen of the preceding S362. RNA sequence-independent hydrogen bond interactions occur between IBD helix B D359 and the ribose of the second *tetraloop* residue.

These interactions with the SRP *tetraloop* optimally position FtsY for formation of the closed complex with the NG domains of the Ffh. Due to steric effects the closed complex detaches from the SRP RNA *tetraloop* in the context of the ribosome (Halic et al., 2006b), as discussed below.

Two conserved sites provide an interface for FtsY at the *distal region* of the SRP RNA

The interaction between FtsY and the SRP RNA at the *distal region* was first described in the structure of *E. coli* FtsY and Ffh in complex with *D. radiodurans* SRP RNA, determined at 3.94Å resolution (PDB identifier 2XXA) in presence of the non-hydrolysable GTP analog GMPPCP (Ataide et al., 2011). The binding interface can be subdivided into two sites, the *docking site* and the *GTPase activation site*, which are separated by one helical turn of the SRP RNA (Figure 2c). Both sites of the RNA are highly conserved among bacteria and across all kingdoms of life (Figure 2, S4).

The interactions at the SRP RNA *docking site* are mediated by helix B and the preceding loop of the FtsY insertion box domain (IBD) and the minor groove of the RNA helix (Figure 2a, Figure S1). Within experimental accuracy, these interactions are equivalent to the previous structure (Figure 4).

However, the RNA backbone in the current structure is partially untwisted and G83 is inserted into the cleft between the G-domains of FtsY and Ffh. Another flipped-out base, C87, occupies a secondary binding site on the surface of FtsY, where it interacts specifically with K406. The untwisted region of the RNA backbone is stabilized by interactions of K399 and R402 with the phosphate groups. In the previous structure, C87 is base-paired to G22 and C86 is flipped out (*E. coli* numbering), occupying the binding site between the two proteins (Ataide et al., 2011). Inspection of the secondary structure diagram of the SRP RNA in this region (Figure 2c) reveals that both conformations may exist at equilibrium.

Interestingly, the same residues of FtsY that mediate *tetraloop* binding also contribute to the *distal interaction* and engage in very similar interactions at the two sites. For example, the hydrogen-bonding pattern between D366 and the unpaired exocyclic amine of G12 echoes the specific recognition at the top of the *tetraloop* base stack. Moreover, salt bridges to the RNA backbone are mediated at both sites by helix 2 residues R402 and K406 (Figure 3a,

Figure 4c). With the exception of the interaction between D366 and the highly conserved G12 (Figure S4), binding of FtsY to the SRP RNA relies on the general electrostatic features (Figure S5) of an RNA helix rather than sequence specific contacts. Mutations in the basic patch residues K399, K402 or K406 (Figure 3 and Figure 4) involved in RNA interactions at the tetraloop and at the distal docking site of the SRP RNA affect both: initial complex formation and the observed GTPase rate. (Figure S6).

The distal region is critical for the stimulation of GTPase activity

Since we now have structural evidence that the RNA may exist in two conformations with either C86 or C87 flipped out of the helix and both C86 (Ataide et al., 2011) and G83 (presented here) have been observed inserted into the GTPase active site in the GMPPCP and GDP-AlF₄ complexes, respectively (Figure 4 and Figure 5a,b), either of these may be implicated in GTPase activation. To explore the functional roles of C86, C87 and G83, we assayed the ability of various SRP RNA mutants to stimulate GTP hydrolysis and translocation.

Deletion as well as any base-substitution of G83 has a deleterious effect on the GTPase activity reducing it to levels observed in absence of RNA (Figure 5c). This demonstrates that not only the presence but also the identity of the base at position 83 is critical for the ability of the SRP RNA to stimulate GTP hydrolysis. The mutations also have a pronounced effect on translocation efficiency, which is strongly reduced compared to wild type RNA (Figure S6). Further reduction of translocation activity is observed in absence of RNA (Shen et al., 2012), consistent with additional roles of the RNA in in the targeting reaction such as accelerating SRP-FtsY assembly via the *tetraloop*.

Base-substitutions of C86 and C87 have opposed effects on GTPase rate: While C87A increases GTPase stimulation, C86A reduces observed rates to those observed in absence of RNA (Figure 5d,e) (Ataide et al., 2011). Neither of the C86 or C87 substitutions have an appreciable effect on translocation efficiency (Figure S6). Deletions of C86, as demonstrated previously (Ataide et al., 2011), or C87 also reduce the ability to stimulate GTPase activity. However, the reduction is not as pronounced, resulting in a 50% lower GTPase rate compared to wild type (Figure 5f).

The existing structural data are consistent with biochemical observations and can be rationalized with the following model (Figure 5a,b): According to the secondary structure diagram (Figure 2c), either C86 or C87 may base pair to G22, as observed in this structure and the previous one (Ataide et al., 2011) (Figure 4b,c). If the flipped-out C is deleted, several interactions with FtsY are lost, which may affect binding affinity. Moreover, a flipped-out base at position 86 sterically impedes access to the GTPase-activating G83 (Figure 4b). Therefore, a substitution that shifts the equilibrium towards a flipped out base at position 87 improves access to G83, and a substitution that shifts the equilibrium towards a flipped out base at position 86 impedes access (Figure 5d,e). This model not only explains the inhibitory effect of the C86 substitutions, but also the stimulatory effect observed for C87 substitutions. Notably, mutation of C86 to G results in only a two-fold reduction in GTPase rate. In addition, double mutations show that the deleterious effects of G83 substitution or deletion on GTPase activation can be compensated by a C86G substitution (Figure 5g). Taken together, these results indicate that a structural state with base 86 inserted into the active site exists in solution, but activation only occurs if the inserted base is a guanosine.

Insertion of G83 rearranges conserved active site residues and water molecules

Compared to the apo-form of FtsY (PDB 1FTS) and the GDP-bound form of Ffh (PDB 1JPJ), both proteins undergo concerted rearrangements during hetero-dimerization in the

presence of non-hydrolysable GTP analogs (PDB 1OKK, 1RJ9) (Egea et al., 2004; Focia et al., 2004). A rigid-body shift of the N domain relative to the conserved core of the G-domain is accompanied by rearrangements of the conserved GTPase sequence motifs G1-G5 (Figure S1) to form an almost symmetrical binding site for two nucleotides at the subunit interface. The 3' OH groups of the FtsY and Ffh-bound nucleotides, which are essential for association and GTP hydrolysis *in trans* donate a hydrogen bond to the γ -phosphate of the opposite nucleotide (Egea et al., 2004; Focia et al., 2004). Large rearrangements occur in the G2/switch 1 (SW1) and G3/switch 2 (SW2) regions of both proteins as well as the G4 and G5 regions of FtsY, relative to the nucleotide-free state (Freyman et al., 1997; Montoya et al., 1997; Egea et al., 2004; Focia et al., 2004). These rearrangements bring several conserved sidechains into the active site: Arginines R141 and R333 of the Ffh- and FtsY G2 regions hydrogen-bond to the α - and γ -phosphate groups. The arrangement of the arginine residues is asymmetric, as they coordinate a single water molecule (C) between the two nucleotides. The G1 aspartate residues 138 and 330 are positioned by hydrogen bonds to a water molecule in the coordination shell of the magnesium ion and a second water molecule (N) close to the γ phosphate, that is thought to act as a nucleophile in GTP hydrolysis (Egea et al., 2004; Focia et al., 2004) (Figure 6a).

In contrast to the large conformational changes during dimerization, the structure of the RNA-bound GTPase heterodimer in complex with RNA and the transition-state analog GDP-AlF₄ does not reveal additional large scale rearrangements (Figure 6b). Nevertheless, the small differences between the structure determined here and the structures of the NG heterodimer in presence of the ground state analogs GMPPCP (PDB 1RJ9, 1OKK) provide important clues to the mechanism of RNA-mediated GTPase activation (Egea et al., 2004; Focia et al., 2004). The active site region of the structure presented here - including the flipped-out base, ligands and water molecules - is well defined, with temperature factors significantly below the average of the structure, also for the observed solvent molecules (Table 1). The flipped-out RNA base G83 reaches into a binding pocket formed predominantly by residues of FtsY in the vicinity of the γ -phosphate (mimic) of the FtsY-bound nucleotide. G83 is stacked between FtsY F332 (G2/SW1 motif) and L393 (helix 1). The 2' hydroxyl group of G83 is within hydrogen bonding distance of FtsY E396, which is in turn positioned by R386 of the FtsY G3 region, and the 5' phosphate of G83 accepts a hydrogen bond from K278 of Ffh. In addition to these stacking- and backbone interactions, G83 forms a Watson-Crick-like hydrogen bond pattern with residues from FtsY and Ffh that is specific for the guanine base: The exocyclic N2 amine donates a hydrogen bond to the backbone carbonyl of FtsY G385 (G3/SW2 motif), the cyclic N1 amine donates a hydrogen bond to the carboxyl group of E277 in the Ffh G5 motif (closing loop) and the O6 carbonyl group of G83 interacts with the main chain amine of Ffh E277 via a bridging water molecule. Thus, the insertion of the flipped-out base may stabilize a catalytically active conformation of the FtsY active site motifs G2 and G3, as well as the Ffh G5 motif. In the light of the specificity of the interaction, it is not surprising that G83 is completely conserved in bacterial SRP RNA sequences (Figure 6c). Moreover, a conserved, unpaired guanine can be identified at the corresponding position of archaeal or eukaryotic SRP RNA sequences (Andersen et al., 2006; Hainzl et al., 2007) (Figure S4).

Compared to the GMPPCP bound state (Figure 6a), the sidechain of Ffh E277 is positioned by hydrogen bonds to G83 and the 3' hydroxyl group of the Ffh-bound nucleotide to interact with the auxiliary (A) water molecule in the activated state (Figure 6b). The auxiliary water in turn hydrogen bonds to the nucleophilic water (N) and the water molecules concertedly move closer to the γ phosphate position occupied by the AlF₄ (Figure 6a,b). As a result, the auxiliary water molecule is exposed to the carboxyl groups of the E277 side chain and the backbone carbonyl of FtsY G385 (G3/SW2 motif). To investigate the role of this residue in propagating the signal from the bound RNA to trigger GTP hydrolysis we have introduced

mutations at this position and measured the rates of RNA-stimulated GTP hydrolysis (Figure 6d,e). The E277A and E277W mutations reduce relative GTPase rate below levels observed in absence of G83, and even the conservative E277Q mutation leads to a fivefold reduction. An essential role of E277 is consistent with the observation that E277 is highly conserved in bacteria (Figure 6d) and across kingdoms (Figure S3b). These results suggest that the insertion of G83 positions E277 of Ffh to polarize the auxiliary water molecule (A) allowing it to partially accept a proton from the nucleophilic water, therefore activating it for attack on the γ -phosphate of the FtsY-bound nucleotide (Figure 6b).

Since the interaction with the flipped-out base of the SRP RNA occurs specifically on one side of the GTPase heterodimer (Figure S5), GTP hydrolysis is likely to begin with the FtsY bound nucleotide. This is supported by the observation that the chemical environment of the auxiliary water molecule near the Ffh bound nucleotide is significantly less polarizing (Figure S5e,f). Moreover, the asymmetrically positioned central water molecule (Egea et al., 2004; Focia et al., 2004) links the α - and γ -phosphates of the FtsY-bound nucleotide to FtsY R333 and Ffh R141. This may stabilize the build-up of negative charges during GTP hydrolysis and also provides a possible route for coupling of GTP hydrolysis at the two sites (Powers and Walter, 1995, Shan et al. 2004).

Discussion

Membrane protein targeting by SRP requires the spatial and temporal coordination of several events (Figure 7b): Initially, SRP binds to the ribosome near the exit tunnel via interactions of the Ffh NG domain with ribosomal protein L23 (Gu et al., 2003) and the Ffh M-domain recognizes the emerging signal sequence (Zopf et al., 1990; Schaffitzel et al., 2006; Halic et al., 2006a). Upon signal sequence binding the SRP molecule adopts a discrete orientation (Estrozi et al., 2011), in which the *tetraloop* of the RNA binds the FtsY and orients it for GTP-dependent association with Ffh, which recruits the RNC to the membrane (Batey et al., 2000; Peluso et al., 2000; Bradshaw et al., 2009). Next, the Ffh and FtsY NG domain complex is detached from the tunnel exit and the *tetraloop* (Halic et al., 2006b) freeing up the L23 region of the ribosome for binding of the translocon (Schaffitzel et al., 2006; Halic et al., 2006a; 2006b). Translocon binding enables repositioning of the NG heterodimer to the *distal region* of the SRP RNA where GTP hydrolysis is triggered, leading to the disassembly of the Ffh:FtsY complex (Ataide et al., 2011; Shen et al., 2012).

Although we have an increasingly accurate mechanistic understanding of this process, several important questions remain; i) How does the SRP RNA position FtsY to accelerate the association with Ffh? ii) Why does the closed complex dissociate from the *tetraloop*? iii) How does the RNA stimulate GTPase activity? iv) How is RNA-mediated GTP hydrolysis delayed until the RNC has been transferred to the translocon?

Interactions with the SRP RNA *tetraloop* position FtsY for binding to Ffh

Biochemical data and cryo-EM show that the Ffh:FtsY complex initially assembles on the *tetraloop* of the SRP RNA and departs from it upon formation of a tight GTP activated heterodimer. However, in the absence of the ribosome the activated complex visualized in this study has maintained interactions with the *tetraloop*. Several lines of evidence suggest that the observed contacts are representative for the initial interactions between FtsY and the *tetraloop*, which occur during RNC recruitment to the membrane; i) very similar interactions have been observed for the early complex between FtsY and Ffh visualized on the translating ribosome by cryo-electron microscopy (Estrozi et al., 2011), ii) these interactions are consistent with mutational and biochemical studies (Egea et al., 2004; Spanggaard et al., 2005; Zhang et al., 2008) which identified regions of FtsY responsible for the *tetraloop* binding, iii) the *tetraloop* interaction site (helix 2, IBD helix B) is spatially separated from

the active site and does not change in response to nucleotide binding and dimerization (Montoya et al., 1997; Egea et al., 2004) (PDBs 1F7S, 1R39). Therefore, we reason that the interaction of FtsY with the *tetraloop* is independent of the functional state of the GTPase heterodimer.

To obtain further insights into the mechanism of NG heterodimer detachment from the *tetraloop*, our high-resolution structure can be combined with information from previous cryo-EM studies. Superimposing the crystal structure to the ribosome bound SRP RNA (Schaffitzel et al., 2006; Halic et al., 2006a) reveals that formation of the *tetraloop* bound closed complex would lead to clashes of the Ffh N-domain with the ribosomal surface (Figure S7). Therefore, the GTP-dependent dimerization supports the dissociation from the *tetraloop* in the ribosomal context. This agrees with the reduced affinity of the closed complex for the ribosome (Shan et al., 2007; Zhang et al., 2009) and the detachment of the Ffh NG domain upon receptor binding (Halic et al., 2006b). In agreement with the transient character of the *tetraloop*:FtsY interaction (Shen and Shan, 2010) in the SRP targeting cycle the interaction surface between FtsY and the *tetraloop* is small ($\sim 270 \text{ \AA}^2$) compared to the *distal site* ($\sim 1000 \text{ \AA}^2$) or the M-domain RNA binding site ($\sim 680 \text{ \AA}^2$).

Insertion of SRP RNA G83 into the active site of FtsY triggers GTP hydrolysis

In this work we visualized the activated complex of the GTPase heterodimer bound to the *distal region* of the SRP RNA (Figure 2). The complex is stabilized by the presence of GDP-AlF₄ in the active site, which mimics the transition state of GTP hydrolysis and significantly increases the affinity of the NG heterodimer for the RNA. At 2.6 Å resolution, the structure allows us to characterize the *distal docking site* of FtsY on the SRP RNA (Figure 4) and to deduce how the flipped out nucleotide rearranges the active site to stimulate GTP hydrolysis (Figure 5,6). The flipped out base of G83 inserts on one side of the NG heterodimer into a binding pocket on FtsY and contacts the G2 and G3 motifs of the FtsY active site, close to the γ -phosphate mimic of FtsY bound GTP. Although most interactions involve FtsY residues, G83 also reorients the completely conserved E277 (Figure 6, S3b) provided *in trans* by Ffh to interact with the auxiliary water molecule. Consequently, the auxiliary water molecule is exposed to several carbonyl- and carboxyl groups. This suggests a chemical mechanism in which the auxiliary water molecule partially accepts a proton from the nucleophilic water and consequently activates it for nucleophilic attack on the γ -phosphate of the FtsY-bound nucleotide (Figure 6, Figure 7a). A catalytic mechanism involving a second water molecule has recently been proposed for the related Ras GTPase: Ras residue Q61 occupies a position close to the reoriented Ffh E277 sidechain and has been implicated in the stabilization OH- and H₃O⁺ ions generated in the reaction (Martín-García and Mendieta-Moreno, 2012). In agreement with such a mechanism, two water molecules are also observed in GTP bound Ras (PDB 1CTQ), and the Ras Q61A mutation decreases GTPase activity, while the Q61E mutation increases it (Martín-García and Mendieta-Moreno, 2012).

The asymmetric interaction between the NG heterodimer and the SRP RNA is reflected by the differences in the charge distribution on the surface of the proteins and by sequence differences near the active site (Figure S5). Moreover, the relative positions of the water molecules differ between the two-nucleotide binding sites of the heterodimer. The chemical environment of the auxiliary water molecule at the Ffh-bound nucleotide is significantly less polarizing than at the FtsY-bound nucleotide, indicating that although GTP hydrolysis is coupled (Powers and Walter, 1995), it may be triggered in an asymmetric manner, beginning with the FtsY-bound nucleotide (Figure S5e,f, Figure 7a). As observed previously (Focia et al., 2006), the central water molecule (C) bound at the interface of the NG heterodimers, is positioned asymmetrically relative to the two nucleotides. It bridges the α - and γ -phosphates

of the FtsY-bound nucleotide but does not contact the Ffh-bound nucleotide. The position of the central water relative to the FtsY-bound nucleotide mirrors the position of the Arginine Finger R789 in the complex of Ras with its GTPase activating protein, GAP334 (PDB 1WQ1) (Scheffzek et al., 1997). The central water is within hydrogen bonding distance of the two asymmetrically positioned arginine sidechains of FtsY (R333) and Ffh (R141), suggesting that it plays a similar role in the stabilization of negative charges during GTP hydrolysis (Figure 6, Figure S5, Figure 7a).

The final stage of the SRP targeting process involves GTP hydrolysis followed by the dissociation of the SRP:FtsY complex. To prevent premature release of the cargo, GTP hydrolysis must be delayed until the signal sequence has been handed over to the translocon. Superposition of the Ffh:FtsY heterodimer to the *distal region* of a ribosome-bound SRP RNA (Halic et al., 2006a; Estrozi et al., 2011) reveals that the access to the *GTPase site* with the flipped out G83 is sterically precluded. This is consistent with the observation that RNCs displaying signal sequence inhibit the binding of the GTPase dimer to the *distal site* as well as GTPase activation. The inhibition can be overcome by the addition of the translocon (Shen et al., 2012, Akopian et al., 2013b). Therefore, binding of the translocon to the SRP during signal sequence handover may induce a rotation of the SRP RNA that would allow the insertion of the flipped out G83 base into the GTPase active site of the FtsY:Ffh complex, triggering GTP hydrolysis and complex dissociation (Figure 7b).

Conclusions

The structural investigation combined with biochemical experiments reported in this study reveal the molecular basis of the SRP receptor recruitment to the *tetraloop* of the SRP RNA and the GTPase activation at the *distal site* of the SRP RNA. Since the protein and RNA residues involved in the chemistry of GTPase activation are completely conserved, the identified chemical mechanism is likely to apply to SRP targeting across all kingdoms. Future work on this fascinating and complex system is likely to focus on the remaining mechanistic questions, in especially the role of the translocon during cargo handover.

Experimental Procedures

Protein and RNA expression and purification

The shortened RNA construct was generated by PCR from wild type SRP RNA from *E. coli* and refolded as described previously (Ataide et al., 2011). His-tagged Ffh (residues 1-298) and FtsY (residues 196-498) for crystallization and SEC binding assays were expressed in *E. coli* and purified essentially as described (Ataide et al., 2011). The Ffh:FtsY complex was formed by incubation of equimolar amounts of the two proteins for 3 days at 40°C in complex formation buffer (50mM HEPES-KOH pH7.5, 50mM NaCl, 2mM MgCl₂, 2 mM TCEP) supplemented with 1mM GDP, 20mM NaF and 2mM AlCl₃. The complex was purified on a Mono-Q column as described previously for the SRP:FtsY complex. Full length Ffh for biochemical assays was produced as above and E277A/W/Q mutations were introduced by PCR. The mutations were confirmed by plasmid sequencing and protein mass spectrometry and do not alter the elution properties in analytical SEC. Wild type SRP RNA, and proteins for biochemical assays were expressed and purified as described (Peluso et al., 2001). Mutant SRP RNA was prepared by *in vitro* transcription as previously described (Ataide et al., 2011).

Crystallization conditions and structure determination

Purified Ffh:FtsY NG heterodimers were combined with an equimolar amount of refolded RNA. For crystallization, 1μL of well solution containing 18% PEG 3350, 100mM Bis-Tris

pH 7.2, 140mM NH₄OAc and 10mM MgCl₂, was added to 1μL of sample and crystals were grown by vapor diffusion at 19°C in sitting drops. Crystals were stabilized with 25% glycerol and flash-frozen in liquid nitrogen. Data were collected at the Swiss Light Source, PSI, Villigen on the Beamline X06SA. Data reduction and scaling were performed using the XDS package (Kabsch, 2010). The structure was solved by molecular replacement with a models of the Ffh-FtsY NG heterodimer (PDB 2CNW) using PHASER (McCoy et al., 2007). Iterative rebuilding of the model was performed in COOT (Emsley et al., 2010) and ONO (Jones et al., 1991) with several rounds of XYZ, individual ADP and TLS refinement in PHENIX (Adams et al., 2002). TLS groups were defined automatically and updated manually. NCS restraints were applied between chains A/C and B/D during refinement along with secondary structure restraints to limit distortions at the more flexible periphery of the complex. Small thermal motions are observed for the GTPase centers and protein-RNA interfaces, while the N-domains and the termini of the RNA display increased temperature factors. All crystallographic figures were generated with Pymol (Schrodinger).

Analytical size exclusion chromatography

Purified Ffh and FtsY NG-domains were combined in equimolar amounts (approx. 300 μM each) and incubated overnight with 1 mM nucleotide analogue (1mM GMPPCP or 1 mM GDP, 2mM AlCl₃ and 4.5 mM NaF) in complex formation buffer (50 mM Hepes pH7.5, 50 mM NaCl, 1 mM TCEP). GDP:AlF₄ bound NG-dimer:RNA complexes formed rapidly at room temperature. All complexes were set up by incubating 5 μM refolded RNA (Ataide et al., 2011) with 2.5-50 μM NG-dimer 10-30 minutes prior to size exclusion chromatography. 50 μL of each complex were injected onto a Superdex 200 3/150 (GE Healthcare) equilibrated in NG-SEC buffer (50 mM Hepes KOH pH 7.5, 150 mM Potassium Acetate, 1mM TCEP and 25 μM of the corresponding nucleotide analogue).

GTPase activity

Measurements of the stimulated GTPase reaction between SRP and FtsY were carried out as described before (Ataide et al., 2011). In general, reactions containing 100 nM Ffh, 200 nM SRP RNA, and varying concentrations of FtsY were initiated by adding 100 μM GTP (doped with γ-³²P-GTP). Reactions were then quenched by 0.75 M KH₂PO₄ (pH 3.3) at different time points. The product (Pi) was separated from unreacted GTP by thin layer chromatography (TLC) and quantified by autoradiography.

Translocation Assays

Efficiency of the co-translational protein targeting and translocation were determined as described (Powers and Walter, 1997; Shan et al., 2007). Reactions contained 10 μL in vitro translation mixtures synthesizing ³⁵S-methionine labeled preprolactin (pPL), to which 67 nM Ffh, 333 nM wildtype or mutant SRP RNA, 333 nM FtsY, and 0.5 eq/μL of salt washed, trypsin digested microsomal membrane was added to a total volume of 15 μL. Reactions were analyzed by SDS-PAGE followed by autoradiography.

Alignments

Alignments were prepared using CLUSTAL W (Larkin et al., 2007) and visualized using ALINE (Bond and Schüttelkopf, 2009).

Accession numbers

The coordinates of the structure reported in this paper have been deposited in the Protein Data Bank under ID code 4c7o.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- *Tetraloop* interactions suggest a mechanism of detachment from the ribosome
- The activated complex structure reveals residues that stimulate GTP hydrolysis
- The identified key residues in the SRP RNA and FtsY are universally conserved
- Structural and biochemical data suggest a mechanism of GTPase activation

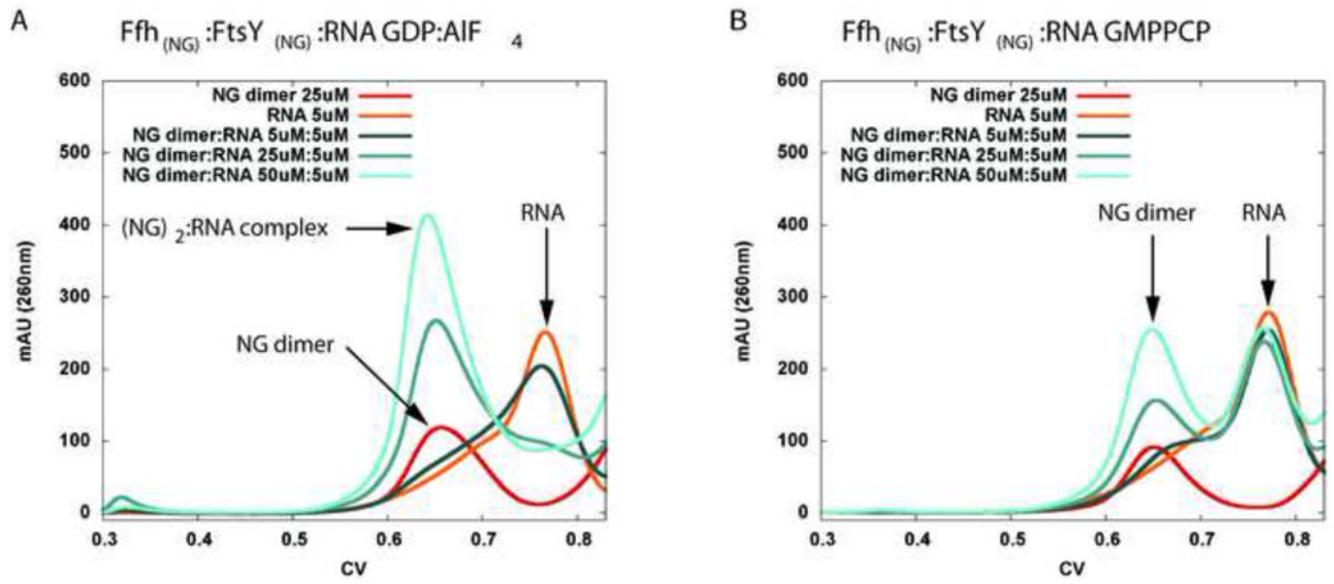


Figure 1. Analytical size exclusion chromatography of NG-heterodimer:RNA complexes
a) Activated complex with transition state analogue GDP:AlF₄ bound. Tenfold molar excess of protein complex over RNA (50 uM : 5 uM) shows full complex formation as seen by a thorough shift of the RNA elution volume. **b)** In contrast, no shift in the RNA or NG-dimer elution volumes is observed if the protein complex is formed with the non-hydrolyzable preactivation state GTP analog GMPPCP. Traces for both proteins and the complex without RNA are shown in figure S2.

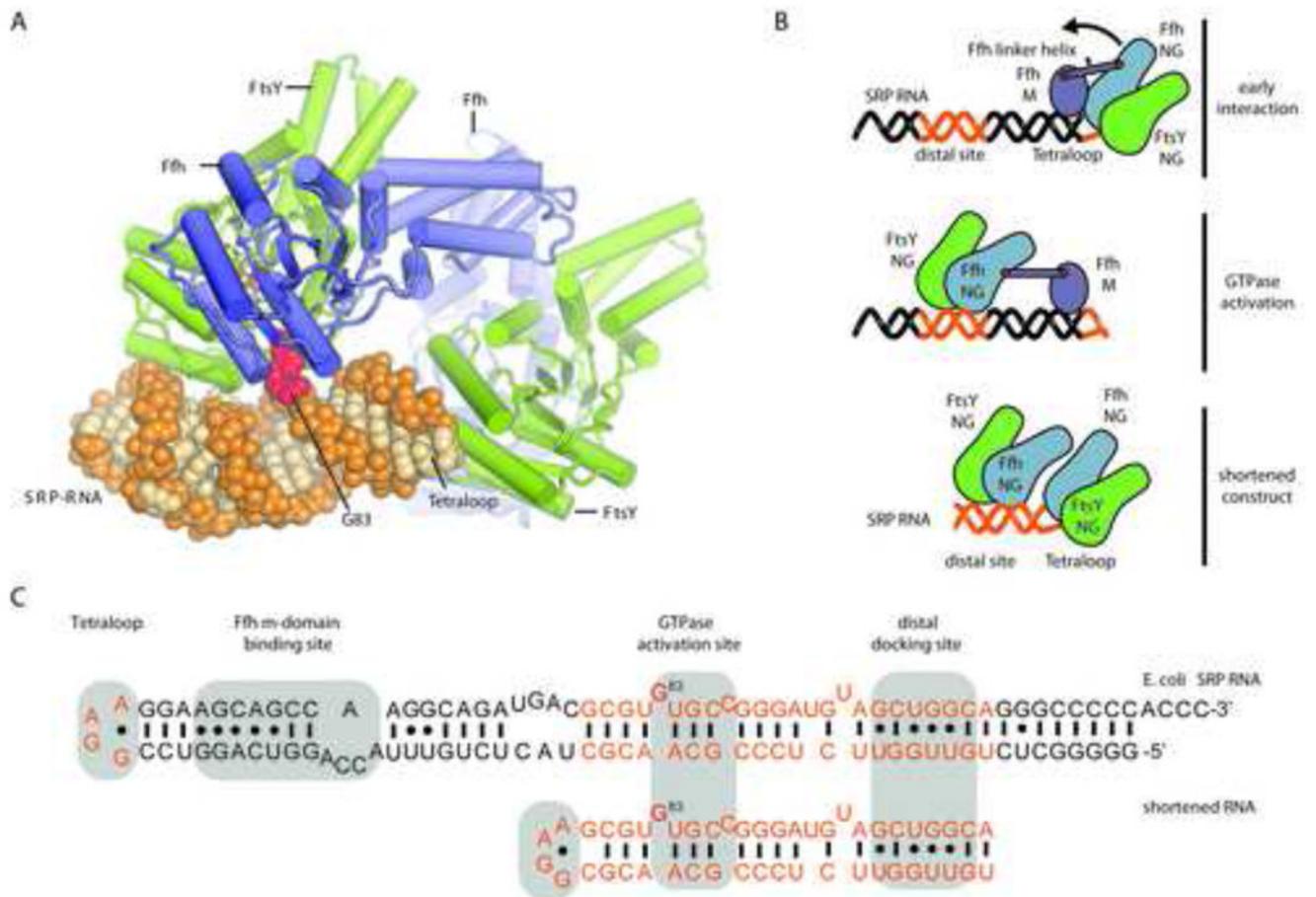


Figure 2. Crystal structure of Ffh:FtsY NG heterodimers bound to truncated SRP RNA in *distal-* and *tetraloop* positions

a) SRP RNA is shown as orange spheres and the GTPase-activating flipped out G83 is highlighted in red. The SRP receptor (SR or FtsY) is shown in green and the Ffh in blue. Two GDP-AlF₄ complexes bound at each dimer interface are shown as sticks, colored by atom. Details of the structural elements of the GTPases are shown in Figure S1 **b)** Schematic representation of the structural rearrangements during substrate recognition and GTPase activation. (top) Initial interactions between SRP and the ribosome are mediated mainly via the N-domain of Ffh and protein L23. FtsY transiently associates with the *tetraloop* (orange) in a GTP-independent fashion, before a tight heterodimer between the homologous NG domains of FtsY and Ffh is formed upon binding of two GTP molecules (middle). The flexible linker tethering M-domain and NG domain of Ffh allows the relocation of the heterodimer to the *distal binding site* (orange) on the SRP RNA, where a flipped-out base stimulates GTPase activity (bottom). **c)** Secondary structure diagrams of *E. coli* SRP RNA and the shortened construct employed in this study. The regions present in the shortened construct are highlighted in orange. *Tetraloop*, *distal docking site* and *GTPase activating site* are shaded in grey. The GTPase-activating G83, which is rotated out of the stack of the helix is highlighted in red.

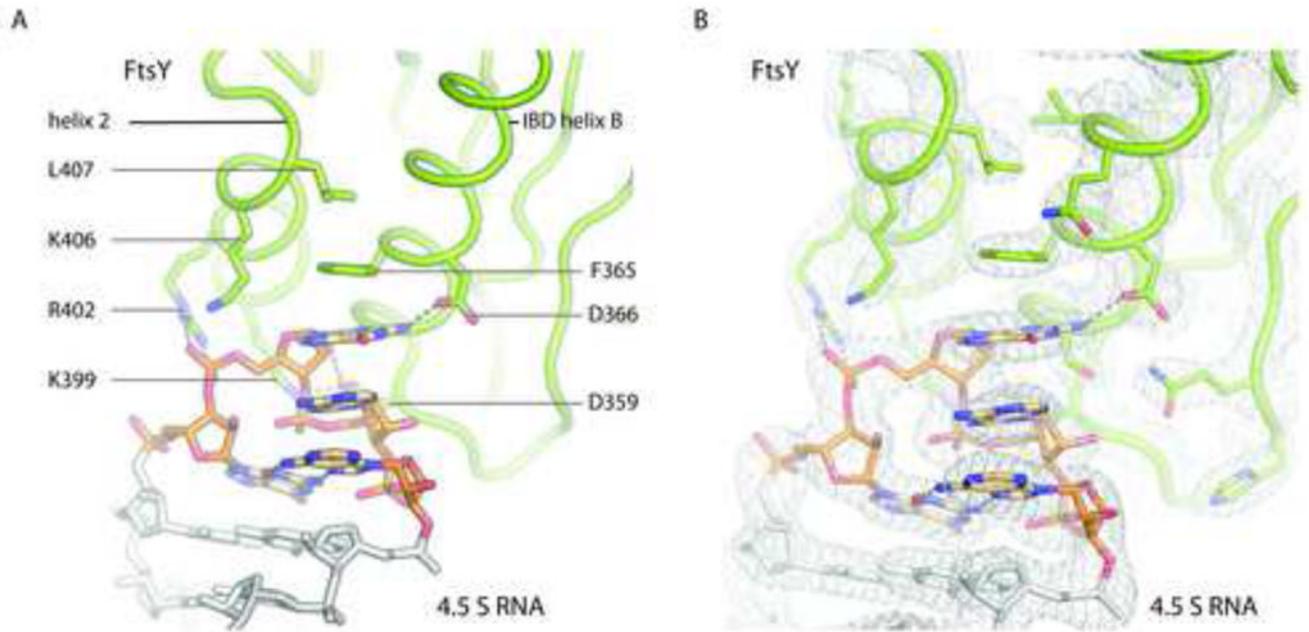


Figure 3. Detailed view of the *tetraloop* FtsY interaction

a) FtsY helix 2 contributes mainly electrostatic interaction with the backbone of the SRP RNA while residues in IBD helix B (Phenylalanine 365 as well as Aspartate 366 and 359) form specific interactions mainly with the base on top of the RNA stack in position to of the GNRA type *tetraloop*. **b)** Electron density at the contact area exemplifies quality of the electron density maps in well-ordered regions. The 2mFo-dFc electron density map shown as grey mesh is countered at 1.5 σ . See also S3, S4, S5 and S6

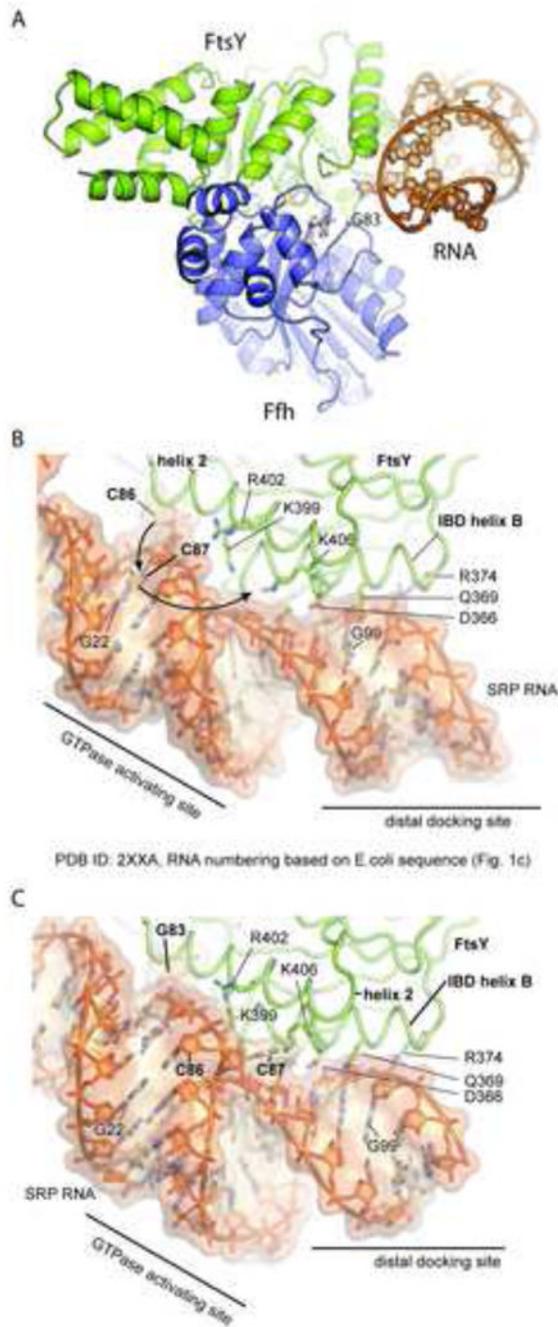


Figure 4. Overview of the distal interaction site

a) Base G83 is sandwiched between the GTPase domains. RNA is almost exclusively bound by FtsY (buried surface area FtsY:RNA $\sim 1000 \text{ \AA}^2$ Ffh:RNA $\sim 90 \text{ \AA}^2$) See also Figure S3 and 4 **b-c)** detailed view of the *distal* interaction site as found in 2xxa (**b**) and 4c7o (**c**). The SRP RNA is mainly interacting with FtsY IBD elements (helix B and to a lesser extent strand B). While the binding of the *distal docking site* nearly identical between the GMPPCP-bound complex shown in **b)** and the structure determined with bound transition state mimic shown in panel **c)**, dramatic differences in the configuration of the *GTPase activating site* are found: An unwinding of approximately 100 degrees of the distal RNA

brings the catalytic base (G83) close to the active site. This RNA conformation also orients the bulge region around bases C86 and C87 in the proximity of the secondary nucleotide-binding site of FtsY allowing C87 to bind into the pocket formed by residues K406, F365 and E396.

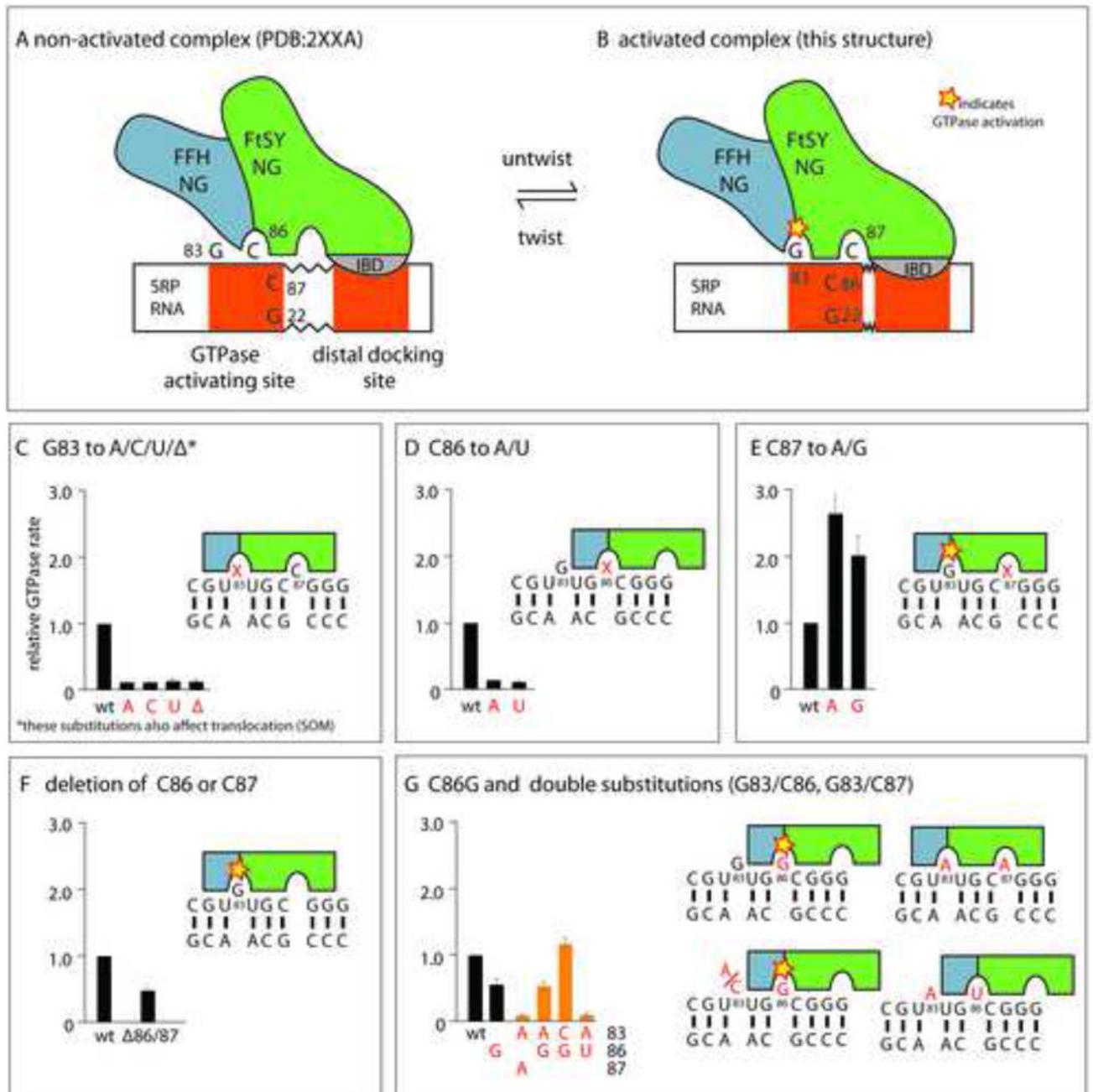


Figure 5. Schematic representation of GTPase:RNA binding modes

a) non-activated and **b)** the activated SRP-FtsY complex. In the activated complex presented here, the RNA is slightly untwisted, and as a result, G83 is inserted into the active site, rather than C86, as observed previously, while the interaction at the *distal docking site* is virtually identical. **c)** Biochemical characterization demonstrates that the presence and identity of G83 is crucial for GTPase activation and also for translocation (see Figure S6). **d)** C86 A or U substitutions strongly reduce GTPase activation, consistent with the observation that a flipped out base at this position impedes access to G83 (Figure 2,3). **e)** In contrast to C86 A or U substitutions, C87A and G substitutions *increase* GTPase activation. A flipped out base at position 87 leaves G22 available for basepairing to C86 and therefore

improves accessibility of G83. **f)** Deletion of either C86 or C87 results in a modest reduction of GTPase rate, in agreement with the loss of interaction surface area (Figure 2c) that weakens the interaction between RNA and FtsY. **g)** Substitution of C86 with a guanosine only reduces GTPase activation moderately, compared to the C86 A or U substitution panel (d). The C86G substitution rescues GTPase activation by SRP RNA with a G83 substitution (panel c), thus indicating that a flipped-out G86 may insert into the active site to stimulate GTP hydrolysis. Data represent mean \pm SD (c-f: n=3, g: n=2)

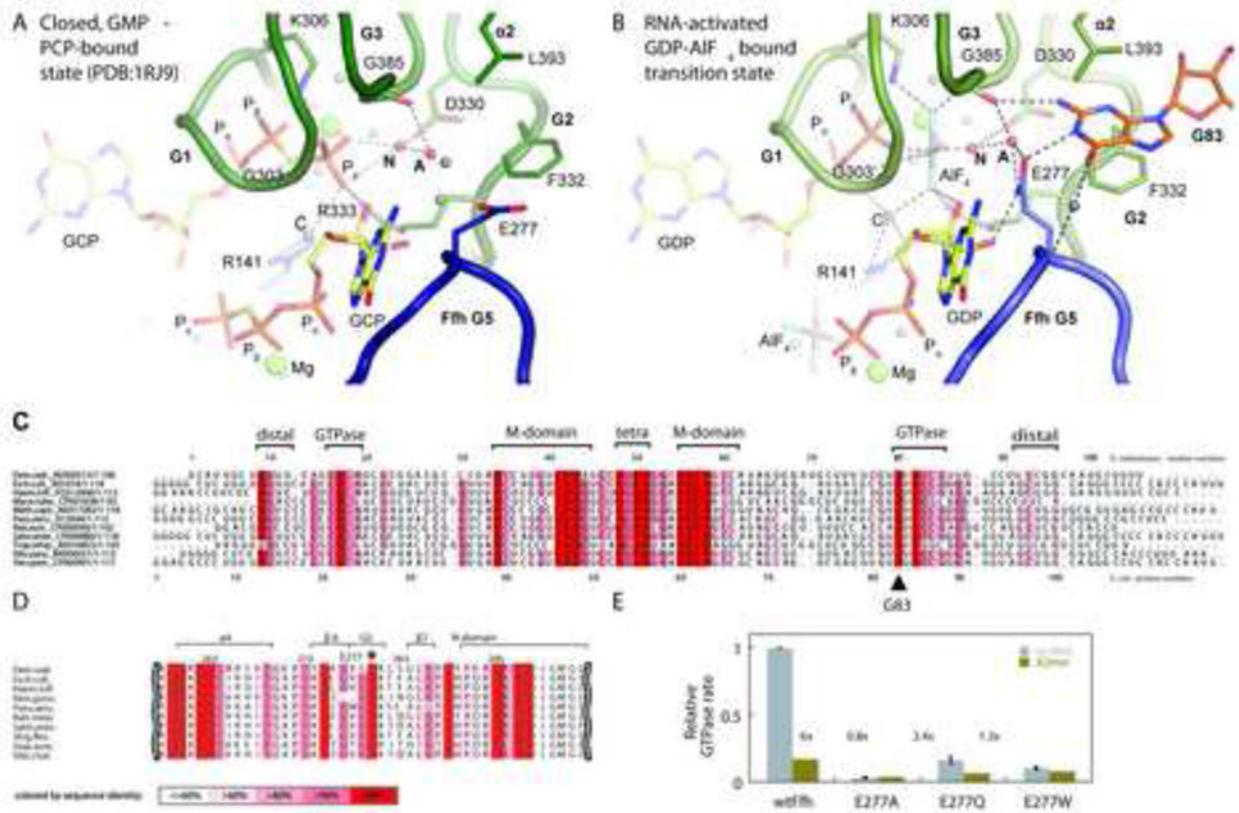


Figure 6. Active site comparison and conservation levels of SRP

a) Closeup of the active site in the GMPPCP bound form (PDB 1RJ9). Glutamate 277 in the closing loop points away from the active chamber. The closing loop of Ffh is shown as a dark blue cartoon, and the P-loop (G1), SW1 (G2) and SW2 (G3) regions of FtsY are shown as a green cartoon (see also S1 and S5). The nucleophilic (N) and auxiliary (A) water molecules implicated in GTP hydrolysis (Focia et al., 2006) are highlighted in red. Dashed lines represent hydrogen bonds. **b)** RNA bound complex with GDP:AlF₄. The flipped out base G83 of the SRP RNA makes specific contacts with FtsY and Ffh residues, organizing a network of hydrogen bonds that finally activates the nucleophilic water (N) for hydrolysis of the FtsY bound GTP. **c)** Sequence alignment of bacterial SRP RNAs. Conservation is indicated by color intensity, based on an alignment of more bacterial sequences (Figure S4). G83 (*E. coli* numbering) and several other residues on both strands of the GTPase-activating region are highly conserved. Significant conservation is also observed for residues of the *distal binding site*. **d)** Alignment of bacterial Ffh sequences. The universally conserved E277 (*E. coli* gene numbering) is indicated by (*) and has 100% sequence identity, also across sequences from different kingdoms (full alignment in figure S3b) **e)** Effects of Ffh E277 mutations on stimulated GTP hydrolysis by SRP. E277A and E277W substitutions reduce GTPase rate below levels observed in absence of the activating G83, and even the conservative E277Q mutation reduces GTPase rate approximately fivefold. The difference in activity observed in presence of full length (grey bars) or truncated SRP RNA (Ataide et al., 2011) lacking the *distal binding site* (green bars) shows that the Ffh mutants cannot be effectively stimulated by SRP RNA. Shortened RNA is assayed once; data for wt RNA represent the mean of two experiments +/- SD.

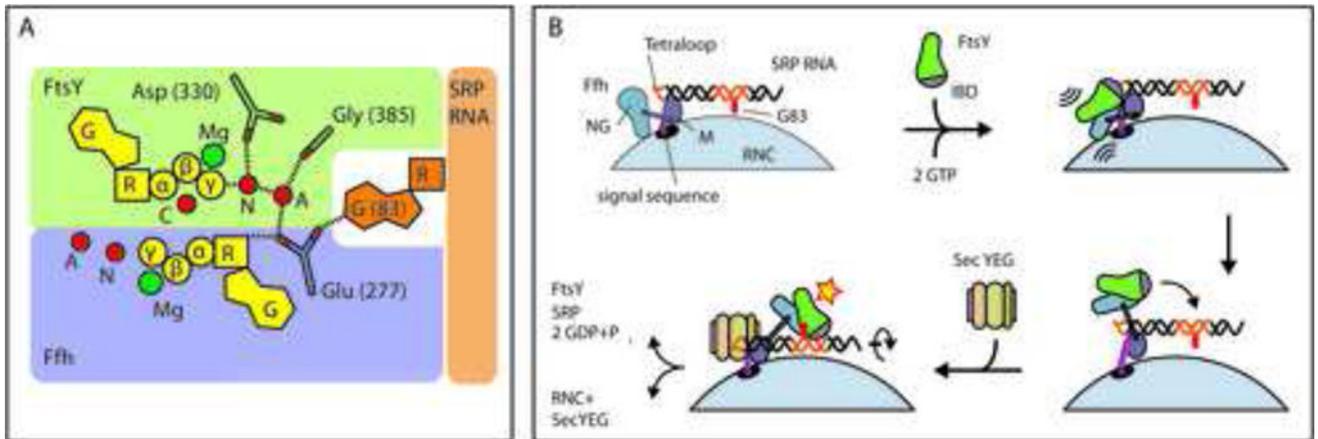


Figure 7. a) Schematic representation of GTPase activation and b) steric regulation in the SRP cycle

(a) Insertion of G83 reorients Glu 277 of Ffh and repositions active site water molecules. The auxiliary water molecule (A) is polarized by interactions with several carboxyl and carbonyl groups in its environment and may therefore partially accept a proton from the nucleophilic water molecule (N) activating it for attack on the γ -phosphate. The central water molecule (C) bridges the α - and γ -phosphates of the FtsY-bound nucleotide and stabilizes the negative charges. (b)(1) SRP recognizes the signal sequence on the nascent peptide chain emerging from the RNC via the M-domain of Ffh. (2) FtsY binds to the SRP RNA *tetraloop* and is oriented for interaction with Ffh. The formation of the closed, GTP-bound complex supports the detachment of the NG heterodimer from the *tetraloop*, see also S7 (3) However, access to the *distal site* is blocked in absence of SecYEG (Ataide et al., 2011, Shen et al., 2012)., (4) Binding of SecYEG may induce a rotation of the SRP RNA that facilitates SRP remodeling and insertion of the catalytic G83 into the active site of FtsY

Table 1
Crystallographic data collection and refinement statistics

Wavelength (Å)	1.00
Resolution range (Å)	49.22 - 2.6 (2.693 - 2.6)
Space group	P 21 21 21
Unit cell	56.72 166.33 154.59 90 90 90
Total reflections	195365
Unique reflections	45637 (4506)
Multiplicity	4.28
Completeness (%)	99.32 (99.21)
Mean I/sigma(I)	13.38 (2.77)
Wilson B-factor	61.98
R-factor	0.1632 (0.2701)
R-free	0.2255 (0.3576)
Number of atoms	10375
macromolecules	9709
ligands	164
water	501
Protein residues	1214
RMS(bonds)	0.010
RMS(angles)	1.34
Ramachandran favored (%)	96
Ramachandran outliers (%)	0.088
Clashscore	17.94
Average B-factor	87.50
macromolecules	89.10
solvent	67.70

* statistics for the highest-resolution shell are shown in parentheses.