Review

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Structure, dynamics and interactions of large SRP variants

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Abstract: Co-translational protein targeting to membranes relies on the signal recognition particle (SRP) system consisting of a cytosolic ribonucleoprotein complex and its membrane-associated receptor. SRP recognizes N-terminal cleavable signals or signal anchor sequences, retards translation, and delivers ribosome-nascent chain complexes (RNCs) to vacant translocation channels in the target membrane. While our mechanistic understanding is well advanced for the small bacterial systems it lags behind for the large bacterial, archaeal and eukaryotic SRP variants including an Alu and an S domain. Here we describe recent advances on structural and functional insights in domain architecture, particle dynamics and interplay with RNCs and translocon and GTP-dependent regulation of co-translational protein targeting stimulated by SRP RNA.

Keywords: co-translational targeting of ribosome-nascent chain complexes (RNCs); dynamics of macromolecular complexes; protein-RNA and RNA-RNA interactions; signal recognition particle (SRP); X-ray and cryo-EM structures.

Introduction

Targeting of membrane or secretory proteins to the endoplasmic reticulum (ER) membrane in eukaryotes or the plasma membrane in prokaryotes is the initial and crucial step in protein biogenesis and homeostasis. The major route of co-translational targeting of nascent

polypeptide chains bearing an N-terminal signal depends on the universally conserved signal recognition particle (SRP) (Cross et al., 2009; Grudnik et al., 2009; Elvekrog and Walter, 2015; Gupta et al., 2017; Ziehe et al., 2017; Steinberg et al., 2018), while ER targeting of proteins with more C-terminal signals follow the SND or TRC40/GET pathways (Aviram and Schuldiner, 2017). SRP is a ribonucleoprotein particle with a preserved core and taxon specific extensions (Andersen et al., 2006) that delivers ribosome-nascent chain complexes (RNCs) via interaction with the membrane-bound SRP receptor (SR) to the translocation machinery (the Sec61 translocon in eukaryotes, SecYEG in bacteria) in a GTP-dependent process (Connolly and Gilmore, 1989) (Figure 1A).

While the SRP targeting cycle is studied since the early 1980s (Meyer and Dobberstein, 1980; Walter and Blobel, 1980, 1981b, 1982; Gundelfinger et al., 1983), progress in biological research came along with several ‘SRPrises’ and paradigm shifts. An elaborate network of chaperones, targeting factors and enzymes is now known to compete for emerging nascent chains and to influence SRP specificity and fidelity *in vivo* (Kramer et al., 2009; del Alamo et al., 2011; Ast et al., 2013; Gloge et al., 2014; Chartron et al., 2016). The discrimination of SRP signals has been systematically refined and revealed the importance of hydrophobicity of the N-terminal cleavable signal sequences and the location of transmembrane domains that act as signal anchors (Zheng and Gierasch, 1996; Martoglio and Dobberstein, 1998; Hegde and Bernstein, 2006; del Alamo et al., 2011; Schibich et al., 2016). Current data of proximity-specific ribosome profiling suggest, although debated (Reid and Nicchitta, 2015), that SRP solely targets pioneering RNCs within a polysome that furtheron stay associated with the target membrane (Jan et al., 2014; Chartron et al., 2016). This limited recognition model explains the observation of sub-stoichiometric cellular amounts of SRP (and its receptor) over ribosome concentrations (approx. 1:10 to 1:100 molar ratio) (Jensen and Pedersen, 1994; Ogg and Walter, 1995). Data on translationally stalled ribosomes reported that SRP samples RNCs [‘sampling’ or ‘scanning’ mode (Halic et al., 2004; Wild et al., 2004; Holtkamp et al., 2012; Voorhees and

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Figure 1: Co-translational targeting by the SRP system.

(A) Scheme for the mammalian SRP system and the targeting of RNCs to the translocon in the ER membrane. SRP consists of six proteins (numbered by molecular weight in kDa) bound to SRP RNA acting as scaffold. The Alu domain reaches into the factor binding site (FBS) within the ribosomal 40S/60S subunit interface and the S domain binds to the signal sequence (signal) emerging from the polypeptide exit tunnel (exit). The multi-domain SRP GTPase SRP54 recognizes the signal with its M domain and establishes the GTP-dependent (denoted with T) targeting complex (TC) consisting of its NG domain bound to the homologous NG domain of the SRP receptor SR α . The SRX domain of SR α regulates the membrane-anchored Ras-like SR β GTPase. (B) Composition of the small bacterial SRP system as shown for Gram-negative *E. coli*. SRP consists of the generally conserved core (Ffh: 54 homolog, 4.5S RNA: helices 5 and 8) and the receptor protein FtsY including an unstructured A domain responsible for membrane attachment. (C) The long bacterial SRP of Gram-positive *B. subtilis*. SRP RNA is doubled in length (6S RNA) and consists of the Alu domain (helices 1–5) and the S domain as found in 4.5S RNA. (D) The archaeal SRP system as found for *Sulfolobus solfataricus*. S domain RNA is extended by helix 6 bound to SRP19 that clamps helices 6 and 8 together. SRP RNA helix 7 is missing. (E) Eukaryotic SRP exemplified for the mammalian system. SRP is supplemented with two heterodimers: SRP9/14 within the Alu domain and SRP68/72 within the S domain. SRP RNA helix 1 is missing. The receptor consists of SR α and the membrane-anchored SR β . SRX (X) is a Longin-like domain locking SR β in its GTP-bound form.

Hegde, 2015)] recognizing its targets even before the signal emerges from the ribosome (Bornemann et al., 2008; Berndt et al., 2009) and that information on protein destination, at least in bacteria, might be already encoded within the messenger-RNA (Fluman et al., 2014). However, recent ribosome-profiling data in bacteria indicate that internal transmembrane domains are selectively recognized and that the N-termini of nascent inner membrane proteins are frequently skipped (Schibich et al., 2016). Whether this holds true for eukaryotic SRP is not known. Time-resolved single-molecule fluorescence resonance energy transfer studies (Noriega et al., 2014a,b) revealed rapid and stable SRP binding to RNCs still to rely on the exposure of the signal outside the ribosomal peptide tunnel as originally proposed (Walter et al., 1981). Thus, signal-independent RNC binding (Houben et al., 2005;

Bornemann et al., 2008; Denks et al., 2017) is possible but seems not to be stable. The case might be similar in eukaryotes, where signal-independent SRP preloading of RNCs during membrane targeting was confirmed recently (Chartron et al., 2016). Nearly all structural data on SRP-RNC complexes as obtained by cryo-electron microscopy (cryo-EM) techniques represent the stable ‘targeting’ (or ‘engaged’) mode (Halic et al., 2004, 2006a; Estrozi et al., 2011; Beckert et al., 2015; von Loeffelholz et al., 2015; Jomaa et al., 2016) with an exposed signal and only recently a first structure of a complex in scanning mode has been reported (Voorhees and Hegde, 2015). High resolution X-ray structures of respective scanning complexes are still pending.

Signal sequence recognition has been described to coincide with a retardation of nascent chain elongation

(elongation arrest) that ensures a time-window for faithful targeting (Walter and Blobel, 1981a; Siegel and Walter, 1988c; Bui and Strub, 1999; Beckert et al., 2015). However, these data were acquired mainly for eukaryotic systems using *in vitro* assays, and the mechanism came into question recently (Chartron et al., 2016). In yeast it has been accounted to non-optimal codon usage instead (Pechmann et al., 2014). Elongation arrest by SRP is induced far from the polypeptide exit tunnel with SRP binding into the factor binding site (FBS) in the ribosomal subunit interface (Halic et al., 2004; Beckert et al., 2015; Voorhees and Hegde, 2015) (Figure 1A). For signal recognition and elongation arrest to occur simultaneously SRP has to stretch over the ribosomal surface, which is only possible for eukaryotic, archaeal and a subset of bacterial SRPs bearing a large SRP RNA acting as scaffold for the SRP proteins. However, SRP RNA has additional functions, and for the small bacterial SRP of *Escherichia coli* it has been shown to coordinate conformational dynamics throughout the targeting process and to stimulate GTPase activity present in SRP and SR necessary for recycling the SRP system (Estrozi et al., 2011; Shen et al., 2013b; Voigts-Hoffmann et al., 2013). SRP functions depend on its complex and dynamic interactions with the RNC, the SR, the translocon and the membrane lipid environment. While these data have been reviewed for small SRP systems (Zhang and Shan, 2014; Elvekrog and Walter, 2015), we will focus here on recent developments and unexplored areas of large bacterial and eukaryotic SRP variants.

Small and large SRP variants

SRP complexity significantly increased in evolution (Andersen et al., 2006; Rosenblad et al., 2009). The conserved core of the ribonucleoprotein complex consists of the SRP54 protein (Ffh in bacteria for 54 homolog) bound to an RNA stem-loop structure (helix 8) (Figure 1B) with highest conservation in two internal bulges and the capping tetranucleotide loop (tetraloop). In the simple small SRP variant of Gram-negative *E. coli* the RNA consists of 114 nucleotides (4.5S RNA). SRP54 is a multi-domain SRP GTPase of the SIMIBI (SRP, MinD and BioD) family (Leipe et al., 2002; Bange and Sinning, 2013) consisting of an N-terminal four-helix bundle (N domain), a central GTPase fold (G domain) with a specific insertion box (I-box), and a C-terminal α -helical and methionine-rich domain (M domain). The M domain is responsible for signal sequence recognition in a hydrophobic groove

enriched in methionines (Zopf et al., 1990; Keenan et al., 1998; Rosendal et al., 2003; Janda et al., 2010; Hainzl and Sauer-Eriksson, 2015; Voorhees and Hegde, 2015) and in RNA binding to the conserved internal bulges of helix 8 (Batey et al., 2000) (Figure 1A and B). The N and G domains form a structural unit termed NG domain (Freyman et al., 1997) that is connected to the M domain by a flexible linker helix (Rosendal et al., 2003; Ataide et al., 2011; Hainzl et al., 2011).

In the phylogenetic branch of Gram-positive bacteria, *Bacillus subtilis* SRP retains the same composition albeit the size of SRP RNA is more than doubled (271 nucleotides, 6S RNA) (Figure 1C). This large SRP variant is divided into two functional and flexibly linked domains, the Alu domain comprising the 5' and 3' ends of SRP RNA and the S domain including the SRP core region. The Alu domain is responsible for the elongation arrest activity of SRP by interfering with elongation factor binding to the FBS in the ribosomal subunit interface (Figure 1A) (Halic et al., 2004; Beckert et al., 2015). In the kingdom of Archaea, SRP has evolved into a large variant with a further expanded SRP RNA including in total seven RNA helices (7.7S RNA, helices 1–8, helix 7 is missing) (Figure 1D). Helices 6 and 8 are clamped together by an additional protein, SRP19 (Wild et al., 2001; Kuglstatter et al., 2002).

In all bacteria and archaea, the SRP receptor consists of the membrane-attached and translocon-associated SRP GTPase FtsY (Figure 1B, C and D). The domain architecture of FtsY differs from SRP54, with the M domain being absent, while an N-terminal acidic domain (A domain) is present. The A domain is highly diverse in length and sequence composition (Andersen et al., 2006) and intrinsically disordered (de Leeuw et al., 1997; Montoya et al., 1997; Stjepanovic et al., 2011; Lakomek et al., 2016). It is responsible for membrane attachment as shown for *E. coli* by a lipid-sensing membrane targeting sequence (MTS) (Bahari et al., 2007; Parltitz et al., 2007; Braig et al., 2009; Stjepanovic et al., 2011) and was recently suggested to bind to the translocon (Jomaa et al., 2017). The heterodimeric and quasi-symmetric interaction of the NG domains of SRP54 and FtsY form the 'targeting complex' (TC) (Egea et al., 2004; Focia et al., 2004b; Wild et al., 2016) (Figure 1A). The TC acts as a communication hub between the RNC and the translocon, and nucleotide-dependent regulation of dimer formation controls the SRP cycle. TC formation is accelerated by the presence of signal sequences (Bradshaw et al., 2009) and SRP RNA (Peluso et al., 2000; Neher et al., 2008). The two GTPases reciprocally activate each other, and for the bacterial system they are known to be stimulated by SRP RNA

(Egea et al., 2004; Shen et al., 2013b; Voigts-Hoffmann et al., 2013).

Eukaryotic SRP further diversified and generally acquired four additional protein subunits bound to SRP RNA. In the best characterized mammalian system the SRP9/14 heterodimer stabilizes the Alu domain fold (Weichenrieder et al., 2000, 2001), whereas the large SRP68/72 heterodimer binds to internal bulge-loops of helix 5 and the three-way junction between helices 5, 6 and 8 of the S domain (Menichelli et al., 2007) (Figure 1E). It was only until recently that the entire human SRP could be completely reconstituted *in vitro* and characterized in detail for its kinetic (Lee et al., 2018) and thermodynamic (Wild et al., 2019) interplay with the ribosome. Eukaryotic SRP RNAs (7S RNA, 300 nucleotides for human SRP RNA) lack helix 1 and the lengths of helices 3 and 4 differ significantly among phylogenetic groups. The largest characterized SRP variant is found in fungi with three additional RNA helices of unknown structure and function, adding up to 522 nucleotides in *Saccharomyces cerevisiae*.

Eukaryotic SR is a heterodimer of SR α (FtsY homolog) and the Arf-like SR β GTPase (Tajima et al., 1986) (Figure 1E). In SR α the A domain is replaced by an N-terminal SNARE-like Longin domain (SRX domain) (Schwartz and Blobel, 2003; Schlenker et al., 2006; Jadhav et al., 2015b) connected to the NG domain via a long and charged linker mediating ribosome binding of the receptor (Jadhav et al., 2015a). The SRX domain is responsible for interaction with SR β in a GTP-dependent manner (Legate and Andrews, 2003). SR β is inserted into the membrane by an N-terminal membrane anchor (Ogg et al., 1998).

The Alu domain of bacterial SRP

In accordance with its simplicity, most structural and functional studies have been conducted for SRP systems of Gram-negative bacteria lacking an Alu domain and the elongation arrest function. However, a substantial number of Gram-positive bacteria (*Bacillus*, *Listeria*, *Clostridium*) and at least one Gram-negative bacterium (*Thermotoga maritima*) have a large SRP RNA containing an Alu domain (Regalia et al., 2002). It has been shown that the bacterial SRP Alu domain is capable of slowing down translation in a cell-free translation assay when SRP is bound to an RNC (Beckert et al., 2015). This raises the question why certain groups of bacteria possess an Alu domain while others do not. In case of *Bacillus subtilis*, a reduced amount of extracellular proteases and deficiencies in sporulation have been observed along with decreased growth during the stationary phase when the Alu domain was deleted

(Nishiguchi et al., 1994). At present, equivalent studies of other bacteria such as *Listeria*, which do not form spores, are missing and thus the physiological role of the bacterial Alu domain remains open. In this respect an open question is, if and how bacteria that lack an Alu domain regulate translation arrest. A hypothetical explanation would be that in such bacteria translation arrest is not required as ribosomes translating SRP targets might be permanently associated with the membrane via the SRP-translocon machinery (Bibi, 2012). In such a case, SRP would not be involved in delivering RNCs to the membrane and thus no translation arrest would be required. Instead, targeting of membrane proteins could already occur at the mRNA level as suggested by several studies (Bibi, 2012; Kraut-Cohen et al., 2013). Another possibility could be self-regulation by mRNAs themselves as certain membrane proteins contain sequences that decrease translation efficiency (Fluman et al., 2014). In addition, both scenarios are not mutually exclusive.

The crystal structure of the *B. subtilis* SRP Alu domain and the cryo-EM structure of *B. subtilis* ribosome-bound SRP have shed light on the fold and ribosome binding mechanism of the bacterial SRP Alu domain (Kempf et al., 2014; Beckert et al., 2015) (Figure 2). In contrast to eukaryotes, the bacterial Alu domain does not rely on a protein component to acquire and maintain its native conformation. Instead, extensions of the RNA serve as inbuilt, stabilizing elements that decrease the conformational freedom and enhance the stability of tertiary interactions. In detail, in higher eukaryotes a hinge between the 5' and 3' parts of the Alu domain allows its closing during SRP9/14 attachment in nucleolar assembly of the particle. In bacteria, this hinge is replaced by a three-way junction introduced due the presence of helix 1, thus dramatically reducing the conformational freedom. In all structures solved so far, including examples from all kingdoms of life, the Alu domain is closed and the 5' and 3' parts of the RNA directly interact by a unique minor groove interaction (denoted as minor-saddle motif) (Kempf et al., 2014). In the case of the human Alu domain, it has been reported that SRP9/14 bind to the conserved 'UGUNR' (N: any nucleotide) RNA U-turn and stabilize the loop-loop pseudoknot formed between the loops of helices 3 and 4 (Huck et al., 2004). Of note, the reduction of the UGUNR to a UGUN sequence fingerprint in bacteria results in a loss of the U-turn and thus of the SRP9/14 binding site (Figures 2A, B and 3). The presence of two additional base-pairs in the loop-loop pseudoknot of *B. subtilis* apparently compensates for the absence of a stabilizing protein component. Accordingly, no homologues of SRP9/14 have been found in the genomes of bacteria and archaea. The report that the DNA-binding

Figure 2: Alu domain structures and ribosome interactions.

(A) Crystal structure of the *B. subtilis* SRP Alu domain (PDB code: 4WFL). Prokaryote-specific stabilizing elements (helix 1 and lobe L4.1) are highlighted in light green. The UGUN motif is highlighted in yellow. (B) Model of the human SRP Alu domain based on two crystal structures (PDB codes 1E80 and 1E85). The Alu domain is stabilized by the SRP9/14 heterodimer. The UGUNR motif forms a U-turn recognized by SRP 9/14 and is colored in yellow. (C) Ribosome-binding mode of the bacterial SRP Alu domain. The Alu domain contains stabilizing elements [Helix 1 (H1), junction III_B, colored in light green] that allow folding into the closed conformation in the absence of protein. The Alu domain binds as a rigid body to the ribosomal factor binding site (FBS) and makes exclusive contacts to the large ribosomal 50S subunit [H43/44, L11, and the α -sarcin/ricin loop (SRL)] via the loop-loop pseudoknot. The SRL is contacted by the prokaryote-specific extension L4.2 (highlighted in light green). Tertiary interactions are indicated by red lines. (D) Ribosome-binding mode of the mammalian SRP Alu domain. In mammals, the SRP Alu domain requires the binding of SRP9/14 to fold into its native conformation. It binds to the ribosome by contacting the L11 protein via the loop-loop pseudoknot and by contacting the small ribosomal subunit (40S) via SRP9/14.

protein HU1 (HBSu) is an integral part of an SRP-like particle in *B. subtilis* and binds to the SRP Alu RNA (Nakamura et al., 1999) has not been confirmed. HU1 has not been found associated with the SRP RNA via *in vivo* pull-down assays (Beckert et al., 2015). Taken together, these data suggest that the SRP Alu RNA of bacteria and archaea does not contain protein.

The cryo-EM structure of *B. subtilis* SRP bound to a stalled ribosome revealed a ribosome binding mode of the SRP Alu domain that diverges from the mammalian system (Figure 2C and D) (Beckert et al., 2015). The Alu RNA is inserted like a plug between the tips of helices 43/44 of the ribosomal stalk base and the tip of the α -sarcin/ricin stem-loop (SRL). A renewed analysis of the mammalian system at higher resolution revealed that the Alu RNA interacts with proteins uL10 and uL11 rather than with the tip of helices 43/44 (Voorhees and Hegde, 2015). In addition, no interaction with the SRL is formed due to a missing extension in the loop-loop pseudoknot that has been lost from eukaryotic SRP Alu RNA. Probably as a compensation for less RNA/RNA contacts, SRP9/14 mediates additional contacts with the small ribosomal subunit, which is not approached by the Alu RNA in the bacterial system.

However, while some details of Alu domain-ribosome interaction differ, the Alu domain binds as a rigid body in both systems, and seems able to slow down elongation by a ‘dock-and-block’ mechanism.

Function and diversity of Alu domains

In the previous section, we discussed crucial structural differences between the bacterial and the mammalian SRP Alu domain. While amongst bacteria and archaea, the structure of the Alu domain is rather conserved, there are tremendous differences between certain groups of eukaryotes (Figure 3). In general, when comparing secondary structure predictions metazoa and higher plants seem to possess an Alu domain similar to the well-studied human system. However, especially among unicellular organisms there is a high structural diversity. Among alveolates (e.g. *Plasmodium*) and euglenozoans (e.g. *Trypanosoma*) the length of helices 3 and 4 varies and some organisms might not possess Alu binding proteins (Zhang et al.,

Figure 3: Alu domain diversity.

(A) Schematics of SRP Alu domains for the three kingdoms of life. RNA helices and the conserved UGUNR sequence fingerprint are highlighted. Bacteria miss the last nucleotide (UGUN). (B) Structure predictions for protozoan SRP Alu domains that diverge from the canonical structure of most eukaryotes.

2013). For instance, the Alu domain of trypanosomes has shortened helices 3 and 4 and smaller closing loops. As in the case of prokaryotes, no SRP9/14 proteins could be identified in the respective genomes. Instead, a tRNA-like molecule has been suggested to interact with the SRP Alu RNA in such organisms (Liu et al., 2003). Also in dinoflagellates and perkinsozoa no Alu domain binding proteins could be identified (Zhang et al., 2013).

Other protozoa such as *Plasmodium* have significantly extended helices 3 and 4 with internal loops and very short closing loops. In case of these organisms, SRP9/14 could be identified in the genome and co-purified with other SRP components (Panchal et al., 2014). SRP14 has a significantly larger loop insertion. In the case of ascomycota (e.g. *Schizosaccharomyces pombe*, *S. cerevisiae*) and basidiomycota (e.g. *Cryptococcus neoformans*), helices 3 and 4 are completely lost with only the UGUNR sequence still being present. A homolog of SRP14 has been found in such fungi, and for *S. cerevisiae* and *S. pombe* it has been shown that SRP14 forms a homodimer (Strub et al., 1999; Brooks et al., 2009). As yeast SRP14 does not bind to the Alu domain of higher eukaryotes, the binding mode seems to be different (Strub et al., 1999). In addition to SRP14, another Alu domain binding protein has been identified (SRP21), which is related to SRP9 (Rosenblad et al., 2004). So far, the binding site and function of SRP21 are unknown. Potentially, this third protein component compensates for the loss of helices 3 and 4. To date, we still miss studies that investigate whether such structural adaptations are correlated with functional differences such as varying efficiencies of elongation arrest.

The SRP68/72 heterodimer of the S domain

While SRP9/14 shape the eukaryotic Alu domain, the two α -solenoidal proteins SRP68 and SRP72 forming a heterodimer within the eukaryotic S domain (60% SRP protein mass) are essential for SRP function (Siegel and Walter, 1988a,c). Knockout studies in *S. cerevisiae* and RNAi silencing of SRP68/72 in *Trypanosoma brucei* lead to an accumulation of pre-SRP in the nucleus, which causes a severe growth defect in yeast and is lethal for trypanosomes (Grosshans et al., 2001; Lustig et al., 2005). In addition, it was shown that reconstituted SRP missing the SRP68/72 heterodimer is impaired in *in vitro* translocation assays while the elongation arrest is still working (Siegel and Walter, 1988b). In the same study SRP68/72 binding to the SRP receptor was shown to be independent of SRP54 and the SRP RNA, respectively. A phosphorylation and a caspase-cleavage site were detected in the C-terminal part of SRP72 (Utz et al., 1998). Caspase cleavage occurs during apoptosis and phosphorylation is induced by mitogen-activated protein kinase (MAPK) postulating a regulatory role of SRP72 in the SRP cycle (Arana-Argaez et al., 2010). Subsequently, SRP68/72 has been reported to act as a transcription regulator by binding to histone protein 4, which was the first role of SRP proteins outside the SRP cycle (Li et al., 2012).

While the biogenesis of the eukaryotic SRP is still enigmatic (Massenet, 2019), the incorporation of SRP68/72 on SRP RNA in the nucleolus is known to lead

to conformational changes in the SRP RNA priming it for binding to SRP54 in the cytoplasm (Kuglstatter et al., 2002). SRP68 and SRP72 are able to bind individually to the S domain of SRP RNA indicating independent RNA-binding domains (RBDs) (Lutcke and Dobberstein, 1993; Iakhiaeva et al., 2005). Different splice variants of human SRP68 (four isoforms) and SRP72 (two isoforms) of unknown function are described, with two SRP68 isoforms missing parts of the RBD (UniProt, 2015). The N-terminal SRP68-RBD binds to the three-way junction of helix 5, 6 and 8 as well as to the 5f-loop (Iakhiaeva et al., 2006; Menichelli et al., 2007; Grotwinkel et al., 2014) (Figures 1A and 4A). SRP68-RBD inserts its second α -helix containing multiple arginine residues (arginine-rich motif: ARM) into the major groove of SRP RNA which leads to a 20° bending of helix 5 (Figure 4A and B). SRP72-RBD crawls along the 5e- and 5f-loops as an elongated peptide (Becker et al., 2017). A lysine-rich cluster and a highly conserved protein family motif (SRP72 Pfam) of the SRP72 C-terminus are described to bind to an 11 nucleotide long region of the 5e-loop (Iakhiaeva et al., 2005). The lysine-rich motif stabilizes the hinge region connecting the Alu and S domain SRP RNA while the Pfam motif interacts with the 5e-loop. The strictly conserved tryptophan W₅₇₇ specifically reads out the 5e-loop (Becker et al., 2017) (Figure 4B). The 5e-loop forms an unconventional kink-turn (k-turn) motif with a potassium ion specifically coordinated by oxygens from nucleobases of both connected stems and was therefore denoted as ‘K⁺-turn’ (Becker et al., 2017). The K⁺-turn kinks the SRP RNA by about 50° in a diametric opposing direction compared to classical k-turns (Klein et al., 2001). The kink in SRP RNA seems to be present in all kingdoms of life, despite the absence of SRP68/72 in archaea and bacteria, and has an important role as ‘docking platform’ for the activated targeting complex (see below) as previously validated for bacteria (Shen et al., 2013a) and recently also confirmed for the mammalian system (Kobayashi et al., 2018). SRP72 binding to SRP RNA also stabilizes the adjacent 5f-loop (A231 in human SRP RNA) in a bulged-out conformation (Figure 4B). A positively charged C-terminal helix of SRP72-RBD (C4-helix) interacts with the 28S rRNA at the so-called C4-contact site of the ribosome (Halic et al., 2004) (see below) and as recently confirmed by an improved cryo-EM structure of the mammalian SRP-SRP receptor targeting complex (Kobayashi et al., 2018). It also contains a conserved glutamine residue that might be involved in TC activation in analogy to the third SRP GTPase FlhF (Bange et al., 2011) involved in flagella biosynthesis (Murray and Kazmierczak, 2006). Furthermore, our recent *in vitro* assembly of the human SRP and the thermodynamic characterization

of its ribosome interactions (Wild et al., 2019) revealed an ultrasensitive binding of SRP68/72 indicating an avidity guided process by the multiple binding sites as described above based on the crystal structures.

Figure 4: The SRP68/72 heterodimer.

(A) S domain structure including the RBDs of SRP68 and SRP72 (PDB code: 5M73). SRP19 (red) clamps helices 6 and 8 of SRP RNA (green). SRP68 (brown) binds to the RNA three-way junction between helices 5, 6 and 8, and inserts the ARM into the 5f-loop, which leads to an RNA bending of 20° . SRP72-RBD (sand) crawls along helix 5 from the 5e- to the 5f-loop. The 5e-loop is stabilized by a potassium ion (purple). (B) Zoom-in view on SRP RNA interactions of SRP68 and SRP72. For SRP68-RBD the ARM of helix α_2 and an extended loop in the three-way junction are given. Two bases of the SRP RNA are bulged out (A231 and G113) and stabilized by SRP72-RBD. The 5e-loop is stabilized by a conserved tryptophan and by the potassium ion coordinated by four highlighted bases. The 5e-loop causes an RNA kink of 50° as indicated by dashed lines. The positively charged C4-helix is not involved in SRP RNA binding. (C) The complex between SRP68 and SRP72 PBDs. SRP72 forms a α -solenoid composed of 4.5 tetratricopeptide repeats (TPRs). SRP68-PBD binds as an elongated peptide to the concave interior of SRP72-PBD.

SRP68/72 heterodimer formation occurs by the N-terminal protein-binding domain (PBD) of SRP72 and a highly conserved peptide at the C-terminus of SRP68 (Iakhiaeva et al., 2009). The SRP72-PBD consists of 4.5 tetratricopeptide repeat (TPR) motifs forming an α -solenoid of nine helices. As typical for TPRs (Zeytuni and Zarivach, 2012), the SRP68-PBD accommodates an elongated peptide of the SRP72-PBD in its concave and rather hydrophobic interior (Becker et al., 2017) (Figure 4C). Shortening of TPR3 by one helical turn enables a stronger curvature of the last three helices of SRP72-PBD and establishes tight SRP68-PBD binding as indicated by a high binding affinity (K_D of 33 nM) compared to other TPR domain proteins (Scheufler et al., 2000). The central parts of SRP68 and SRP72 linking the RBDs with the PBDs are predicted as further TPR motifs and apparently provide a scaffolding platform of an extended solenoidal cradle for the activated targeting complex (see below). Deletion of the central TPRs in SRP72 cause a severe growth defect in yeast (van Nues et al., 2008) underlining the importance of SRP72 integrity. However, despite the ‘resolution revolution’ complete SRP68/72 has not been resolved and its placement in a recent cryo-EM structure (Kobayashi et al., 2018) is not unambiguous.

Among the SRP proteins, SRP72 stands out as it is involved in physiological regulation, and apart from SRP54 (Carapito et al., 2017), also in pathological cues. Familial aplastic anemia can be caused by an inherited mutation in the *SRP72* gene resulting in a stop codon at position 354 (Kirwan et al., 2012). This SRP72 variant does not contain the RBD and is functionally impaired. Patients suffer from inefficient hematopoiesis and have an increased risk for leukemia. SRP72 is also involved in the autoimmune disease of systemic lupus erythematosus, which is caused amongst others by developing autoantibodies against the SRP72 phosphorylated C-terminus upon apoptosis (Arana-Argaez et al., 2010). However, the molecular details, and biological and pathological mechanisms correlated with SRP72 (Utz et al., 1998; Hengstman et al., 2006; Kirwan et al., 2012) are just emerging and are still to be explored in depth.

Signal recognition by the M domain of SRP54

The eponymous SRP function of signal recognition is notoriously difficult to describe on a molecular level. Several difficulties add to this obstacle: (i) the hydrophobicity of the signal and of its binding site within the

SRP54 M domain cause oligomerization or aggregation when exposed to solvent; (ii) signal binding takes place in the context of the RNC and high-resolution structures are not available; (iii) the SRP54 M domain is highly flexible; and (iv) signal binding is non-specific.

First structural investigations revealed the presence of a deep groove lined with side chains of hydrophobic residues, which are biased towards methionines predestined for signal binding (Keenan et al., 1998; Clemons et al., 1999). The conserved and rigid core of the M domain consists of an α -helical fold comprising a helix-turn-helix motif with an arginine-rich helix required for binding to the conserved bulge-loops of SRP RNA helix 8 (Batey et al., 2000) (Figure 5A). RNA binding of SRP54, generally the last step of SRP assembly (Politz et al., 2000), occurs via an induced-fit mechanism reshaping the entire S domain RNA in archaea and eukaryotes (Kuglstatter et al., 2002; Wild et al., 2010). Subsequent structures of the entire SRP54 protein revealed the flexible connection of the M domain via a long linker helix (α GM) to the conserved RILGMD sequence fingerprint at the end of the NG domain, and the dynamics within the rigid and variable parts of the M domain upon signal recognition (Rosendal et al., 2003; Janda et al., 2010; Hainzl and Sauer-Eriksson, 2015) (Figure 5B). Recently, we observed a thermodynamically distinct two-step binding mode of SRP54 to a non-translating ribosome with a high affinity binding of the M domain (low nanomolar) and low affinity binding of the NG domain (low micromolar) highlighting the uncoupling of the domains due to the flexible linker (Wild et al., 2019). Finally structures of SRP54 in the presence of an RNC and/or the SRP receptor (Halic et al., 2004; 2006a; Estrozi et al., 2011; Voigts-Hoffmann et al., 2013; Beckert et al., 2015; Voorhees and Hegde, 2015; Jomaa et al., 2016) highlighted the conformational plasticity in the M domain along the SRP cycle. Taken together, the following key features of the M domain seem valid for all kingdoms of life (Figure 5C): (i) the M domain can reach into the funnel of the polypeptide tunnel exit to grab the signal as soon as it emerges; (ii) the signal opens the M domain by displacing the finger loop and C-terminal helices; (iii) the signal inserts into the hydrophobic groove, apparently in promiscuous orientations; (iv) signal binding induces a switch of the SRP-RNC interaction from the scanning to the engaged state with tight binding of the NG domain across the tunnel exit primed for SR interaction; (v) the linker helix α GM acts as a ‘lever arm’ for coordinating signal release and TC relocation from the ‘proximal’ to the ‘distal’ SRP RNA sites; and (vi) translocon and relocated SRP-SR can bind simultaneously to the RNC to assure the direct handover of the signal and therefore

Figure 5: Signal recognition by the SRP54 M domain.

(A) Structure of the SRP core consisting of the SRP54 M domain (54M: blue, bound to SRP RNA, PDB code 1hq1; gray: PDB code 1qb2). The helix-turn-helix (HTH) motif binds to conserved bulge-loops (asym/sym) of SRP RNA helix 8. The RNA tetraloop (T) at the proximal RNA site is solvent exposed. (B) Signal recognition by the M domain. Top panel: closed M domain without signal (PDB code 1qzx); bottom panel: open M domain with bound signal (cyan, PDB code 3kl4). The helix α GM (purple) links the C-terminal M domain to the highly conserved RILGMD motif (pink) of the NG domain. (C) Signal recognition in context of the eukaryotic RNC (PDB code 1jaj; the 60S subunit is shown in gray outline). The M domain is placed at the end of the polypeptide exit tunnel. Two C-terminal helices (α C) and the finger loop complete signal recognition in the hydrophobic binding groove. Helix α GM following the RILGM sequence is not resolved. SRP54NG stretches over the tunnel exit and is bound in between the proximal SRP RNA tetraloop (T, yellow) and the ribosome.

faithful targeting of the nascent chain. However, high resolution X-ray or EM structures and the structure of the quaternary SRP-SR-RNC-translocon complex revealing the molecular mechanism of cargo handover are still pending. A first such complex was reported for *E. coli*, but the SEC-translocon was displaced by 30 Å from the ribosomal tunnel exit (Jomaa et al., 2017). In addition, the obstacles of signal sensing by SRP already in the polypeptide exit tunnel or on the mRNA level remain unexplained.

Regulation of the SRP cycle by SRP GTPases

The SRP GTPases SRP54 (Ffh) and SR α (FtsY), together with FlhF involved in flagella biosynthesis (Murray and Kazmierczak, 2006), constitute their own sub-family within the SIMIBI-class of P-loop (phosphate-binding loop) NTPases reflecting their structural and functional uniqueness (Leipe et al., 2002; Bange and Sinning, 2013). In general, P-loop NTPases are conserved mixed α/β folds and are characterized by the Walker-A motif (P-loop; GxxxxGK[ST] consensus, x: any residue) that forms the major nucleotide-binding determinant and the Walker B-motif (DxxG) with a conserved aspartate involved in magnesium binding. For SRP GTPases five G elements within the G domain involved in nucleotide binding and

hydrolysis are conserved (G1: Walker-A, G2: Walker-B) (Figure 6A). Larger conformational changes during the GTP-GDP transition (GTPase switch cycle) are confined to the G2 and G3 elements, which in the field of small Ras-like GTPases are called switch I and switch II regions, respectively (Wittinghofer and Vetter, 2011). SIMIBI-NTPases (in contrast to the TRAFAC-NTPases) are characterized by a specific Gx[QD]GxGK[ST] G1 element (P-loop) with an additional glycine, a parallel β -strand adjacent to G2, and an insertion-box (I-box between G2 and G3) that adds another parallel β -strand and two α -helices (Leipe et al., 2002). The SRP GTPase switch cycle is unique as large conformational changes in the switch regions have not been observed, and SRP GTPases are characterized by a low affinity for GTP (μ M range). In contrast to most canonical Ras-type G proteins, SRP GTPases are stable in their nucleotide-free state (Rapiejko and Gilmore, 1997). SRP GTPase function is related to dimerisation (targeting complex, TC), which depends on the presence of GTP in both G proteins. Accordingly, SRP GTPases have been classified as GTPases activated by dimerization (GADs) (Gasper et al., 2009). However, while all members of the SIMIBI-family of NTPases form homodimers, SRP54 and SR α form a nearly symmetric heterodimeric TC (see below), which results in molecular ‘matchmaking’ during the assembly of the ribosome-translocon junction as

originally proposed (Walter and Johnson, 1994). Until recently, the only homodimer of an SRP GTPase has been reported for FlhF, which is stable in the GTP bound state (Bange et al., 2007a), while the heterodimeric TCs are not stable with GTP. Notably, we could recently show that upon binding to membrane lipids FtsY of *E. coli* also forms a homodimer (Kempf et al., 2018), but its function within or apart from the SRP pathway is still unknown. However, while the dimer interface is similar to the FlhF homodimer, the FtsY dimer is not stable with GTP and activates GTP hydrolysis. In this respect it is similar to the TCs, which share the same interface of the G domains. The question still remains, why the FlhF homodimer is different from the FtsY homodimer and the TC heterodimers.

A large number of X-ray structures for the bacterial and archaeal SRP GTPases (NG domains) have been determined in different nucleotide-bound states (Freyman et al., 1997; Montoya et al., 1997; Focia et al., 2004a, 2006; Gariani et al., 2006; Gawronski-Salerno et al., 2007; Egea et al., 2008a,b) and also of corresponding bacterial TCs (Egea et al., 2004; Focia et al., 2004b; Gawronski-Salerno and Freymann, 2007). We completed this structural

database by adding structures for the human, archaeal and also chloroplast TC (Wild et al., 2016). These structures revealed the general blueprint of SRP GTPases and phyla-specific adaptations. The TC forms a quasi-symmetric heterodimer with closely interacting G domains and the nucleotides align in a head-to-tail manner in the center of the interface (Figure 6A). The direct contact between the nucleotides across the dimer interface (‘substrate twinning’) plays an important role in reciprocal activation and 3'-desoxy analogues are inactive (Egea et al., 2004). TC formation in bacteria was described to occur in three steps via an GTP-independent ‘early intermediate’ driven by electrostatics, a consolidated GTP-dependent ‘closed’ state with adapted switch regions to a final ‘activated’ state (Figure 6B) (Shen et al., 2013b). Of note, all TC structures represent the same state irrespective of being crystallized with non-hydrolyzable or transition-state analogs, and thus the closed and activated states are structurally indistinguishable. This fact seems in contrast to a proposed transition from the closed to the activated state by repositioning of catalytic residues (Zhang et al., 2009). Upon hydrolysis nucleotide exchange

Figure 6: The targeting complex (TC) of SRP GTPases.

(A) Structure of the NG domains of the human TC (SRP54 in blue, SRα in yellow) stabilized in its closed conformation by non-hydrolyzable GTP-analogues (gray ball-and-sticks, denoted as ‘GTPs’). The SRP GTPase typical G elements are indicated and labeled for SRP54. The I-box within the G domain is highlighted for SRα (orange). Substrate-twinning in the interface is manifested by hydrogen-bonding (solid black lines) between the nucleotides. (B) Re-localization of the bacterial TC from the GTP-independent early open conformation at the proximal SRP RNA site (T: tetraloop of helix 8) to the late GTP-dependent closed conformation bound to the distal site (loops D and E). Loop E introduces a kink into SRP RNA and forms a docking platform for the TC. Activation of GTP-hydrolysis within the TC occurs at the distal site and is stimulated by bulged out nucleotides at loop D of SRP RNA. (C) The distal site of mammalian SRP consists of the 5e- and 5f-loops that are stabilized by SRP68 and SRP72. SRP68 introduces an arginine-rich motif (ARM) into the major groove of the 5f-loop and SRP72 inserts a tryptophan in the 5e-loop and crawls along both loops. The 5e-loop includes two nucleotides (red, A231 and G232 in human SRP RNA) that potentially are bulged-out. The 5e-loop is kinked as loop D in bacterial RNA and is stabilized by a specifically-bound potassium ion (K⁺, purple sphere). (D) The SRP-RNC contact at the distal site (C4-contact). The contact is established by protein-RNA, RNA-RNA and protein-protein interactions. SRP72 is in the SRP-RNC interspace. Ribosomal compounds (gray) include the 3'-terminal three-way junction of 28S rRNA and ribosomal protein L3. The relocated TC docks onto the kinked 5e-loop and is potentially activated (indicated by a star) by the 5f-loop and SRP72.

is fast and apparently does not depend on an external guanine-nucleotide exchange factor (GEF). The GEF function was found to be in-built in SRP GTPases and corresponds to the I-box (Moser et al., 1997). As described in our detailed analysis of TC structures, the I-box modulates the conformation of the preceeding G2 element (switch I) by a magnesium-sensitive switch with an inducible P-loop at the beginning of the I-box (Wild et al., 2016).

A central question in SRP research is how SRP GTPase activation occurs and how it is coupled to the presence of the signal and to SRP cycle regulation. Biochemical analyses (Jagath et al., 2001; Shen and Shan, 2010) together with X-ray and cryo-EM structures of bacterial and recently also of mammalian TCs in complex with SRP RNA (Ataide et al., 2011; Voigts-Hoffmann et al., 2013) and the RNC (Estrozi et al., 2011; Jomaa et al., 2016; Kobayashi et al., 2018) revealed two alternative RNA binding sites for the G domains on the RNA (Figure 6B): an initial ‘proximal’ binding site independent of GTP-binding on top of the tetraloop of helix 8 [the early TC intermediate (Zhang et al., 2011)] and a late ‘distal’ binding site about 100 Å away on internal bulge-loops (loops D and E) on helix 5 (the activated state). TC closure apparently occurs at the proximal site upon GTP binding resulting in the detachment of the TC from the ribosomal surface. The relocation of the TC to the distal site is crucial for translocon docking of the RNC and for activation of GTP hydrolysis within the TC (Kuhn et al., 2015). Hydrolysis, as described for bacteria, occurs via the rare case of an enzyme being activated by RNA. In brief, a bulged-out single guanine (G83 in *E. coli*) is placed on top of the G2 element of FtsY and a glutamate of the adjacent G5 element of Ffh (Voigts-Hoffmann et al., 2013), thus shielding the glutamate and triggering hydrolysis (presumably in FtsY first) via two buried water molecules. Of note, the distal site is also responsible for 4.5S RNA binding to 23S rRNA of the RNC as shown by cross-linking studies (Rinke-Appel et al., 2002), however, this SRP RNA – ribosome contact is lost upon FtsY binding as revealed by several cryo-EM studies (von Loeffelholz et al., 2015; Jomaa et al., 2016). Dissociation is necessary to avoid steric clashes between the relocated TC and the ribosome. However, molecular details of this SRP RNA – ribosome contact remain unresolved.

While the multiple functions of the distal SRP RNA site are validated for the small SRP variant of *E. coli*, conservation is not evident for the other kingdoms of life bearing a large SRP RNA and additional protein subunits. Especially in eukaryotic SRP the scenario is more complex, as the SRP68/72 heterodimer locates at the distal RNA site and SR includes the Arf-like SRβ GTPase, which might also bind at this site (Halic et al., 2006b). The TC structures

from all kingdoms of life set the basis for an inter-phyla analysis of conservation and adaptation within the TC and its putative regulation by SRP RNA (Wild et al., 2016). The generality of the heterodimeric arrangement, surface charge patterns and of the G elements and their conformations revealed the preservation of the core machinery of co-translational protein targeting suggestive for very similar mechanisms of complex regulation. Especially the binding sites for SRP RNA and its structural connection to the active center was found to be conserved. Chloroplast TC of *Arabidopsis thaliana* exposes an altered surface charge pattern in line with the absence of SRP RNA in higher plants.

The distal site within eukaryotic SRP RNA corresponds to the 5e- and 5f-loops (Figure 6C). The potassium-stabilized 5e-loop introduces a kink into SRP RNA that is conserved in SRP RNA structures solved for all kingdoms of life (Becker et al., 2017). In bacteria, the kink of the respective loop E (potassium-independent) has been found to act as the crucial docking platform for the activated TC (Shen et al., 2013a). The 5f-loop contains two unpaired nucleotides that can potentially be bulged-out (A231 and G232 in human SRP) in contrast to a single guanine nucleotide in bacteria. For the human SRP it has been shown that the 5f-loop is remodeled by SRP68, which inserts the arginine-rich motif (ARM) into the major groove and pushes the nucleotides out (Grotwinkel et al., 2014). This function seems conserved in all eukaryotic SRP systems according to sequence conservation (Andersen et al., 2006). The 5f-loop is further modulated by the C-terminal tail of SRP72, which stabilizes the bulged-out position of A231 and thus presents the nucleotide base to the 3'-terminal three-way junction of 28S rRNA. SRP72 docks the SRP RNA distal region onto the ribosomal surface (C4-contact) (Becker et al., 2017) (Figure 6C and D). In contrast to the small bacterial SRP, the C4-contact is not broken upon SR binding to RNC-bound mammalian SRP (Halic et al., 2006a,b). Here, the SRP S domain rotates up to 20° in respect to the ribosome upon SR binding. Rotation implies breaking the interaction between the 5f-loop and 28S rRNA, and thus the bulged-out bases of the 5f-loop of SRP RNA are free for TC binding in its activating position (Figure 6D). The RNA-RNA contact is however maintained, as an adjacent bulged-out nucleotide (G113 in human SRP RNA) rotates into place. Thus, while small SRPs dissociate from the ribosome for TC activation, large SRPs have evolved a more sophisticated rotational mechanism that apparently constitutes an important ‘checkpoint’ of co-translational targeting. This mechanism was nicely confirmed by the first structure of the ribosome-bound mammalian pre-handover SRP-SR complex (Kobayashi et al., 2018), which suggested that

only the bulged-out G232 complements the active site of the TC. Interestingly, the study revealed that in contrast to bacteria, GTP-hydrolysis is delayed at this stage. The molecular details of this delay are still not understood.

Of note, the very C-terminal tail of SRP72 is intrinsically disordered, and *in vivo* it is phosphorylated on serine residues and cleaved during apoptosis thus shutting-down protein translocation (Utz et al., 1998). The cleavage site is next to the C4-contact region and locates directly to the activated TC bound to the distal SRP RNA site. Therefore, SRP72 integrity and phosphorylation seem to constitute a crucial checkpoint beyond co-translational targeting and influence the cell fate in health and disease.

The blank spots of SRP research

The definition of regulatory fidelity checkpoints has become more and more popular in SRP research (Bange et al., 2007b; Zhang and Shan, 2014; Kobayashi et al., 2018). These checkpoints are thought to kinetically and thermodynamically control the transitions between functional states of co-translational targeting by conformational changes within the participating components. The main problem of a physiologically relevant characterization of the SRP cycle is the complex interplay between the RNC, SRP, SR, the translocon and the membrane, as well as with competing factors in the crowded cellular environment of RNC and translocon. Therefore, all *in vitro* studies are to be viewed with caution as they are limited by a reduced set of components or necessary system simplifications. With the advent of fluorescence techniques, a plethora of quantitative data accumulated mainly for the bacterial system (Saraogi et al., 2014). As the bacterial system can functionally replace the mammalian homologs *in vitro* it is regarded as model system for SRP research (Powers and Walter, 1997). This observation underlines the remarkable evolutionary conservation of the SRP core consisting of the SRP GTPases and SRP RNA helix 8.

The identified checkpoints within the bacterial SRP core concern: (1) specific cargo recognition and corresponding intramolecular transitions of SRP54/Ffh from the sampling/scanning to the targeting/engaged mode, (2) elongation arrest by spatial interference of the Alu domain (when present) with the FBS, (3) early GTP-independent TC formation at the proximal site of SRP RNA at the membrane, (4) structural consolidation into the GTP-dependent closed TC and dissociation from the SRP RNA proximal site, (5) translocon docking and displacement of the TC to the distal site of SRP RNA, and (6) activation of

the TC by the distal site of SRP RNA including the dissociation of SRP RNA from the ribosome. The ‘blank spot’ our mechanistic understanding concerns how signal release into the translocon is achieved (checkpoint five) and coordinated with GTP-hydrolysis as final checkpoint of faithful targeting. However, the structure of a first RNC-SRP-SR-translocon intermediate (Jomaa et al., 2017) rather opened new questions than settling the case, as the translocon was found away from the canonical site at the ribosomal tunnel exit, which seems to object to a direct signal handover.

While small bacterial SRP can be regarded as model for the general mechanism of targeting RNCs to the translocon, it is evident that additional layers of complexity control large SRP systems with some of them still to be unveiled (Figure 7). Checkpoints one and two are structurally validated for the eukaryotic SRP system by cross-linking (Pool et al., 2002) and cryo-EM data (Halic et al., 2004, 2006a). The formation of an early TC complex (checkpoint three) is also likely to occur in both archaeal and eukaryotic SRP (Halic et al., 2006a; Hainzl and Sauer-Eriksson, 2015; Wild et al., 2016). Sec61 translocon docking is structurally incompatible with TC binding to the proximal site as proven by cryo-EM studies (Becker et al., 2009; Gogala et al., 2014; Voorhees et al., 2014; Voorhees and Hegde, 2016). Whether the Sec61 translocon actively displaces the TC (checkpoint four) like shown for bacteria (Akopian et al., 2013) is however not known and signal handover (checkpoint five) is still enigmatic.

For eukaryotic SRP the ‘blank spots’ include also the description of TC activation at the distal site (checkpoint six) (Figure 7), where the eukaryote-specific SRP68/72 heterodimer binds and stimulates SRP structure and function. Recent structural data on human TC and S domain complexes including the RBDs of SRP68 and SRP72, and their placement in cryo-EM reconstructions, revealed three basic items (Grotwinkel et al., 2014; Becker et al., 2017; Kobayashi et al., 2018): (1) SRP68 and SRP72 remodel the distal site (5e- and 5f-loop) of SRP RNA; (2) the interaction of the distal site with the ribosome is significantly extended by SRP72 and is not broken upon SR binding; and (3) the stimulation of the TC is likely to occur by bulged-out nucleotides within the 5f-loop. However, the 5f-loop *per se* is not activating (Kobayashi et al., 2018) and large parts of SRP68/72, their binding to the activated TC, and their placement on the ribosome are unknown. Thus, it has not been possible so far to dissect the mechanism of activation of GTP hydrolysis for the eukaryotic system.

Likewise, the function of the Arf-like SR β GTPase and its integration into the SRP cycle remains enigmatic. The structural basis of its GTP-dependent interaction

Figure 7: Checkpoints within the SRP cycle.

Regulatory checkpoints as validated for the bacterial system are transferred and expanded for the mammalian SRP. Coloring and view as in Figure 1A. The checkpoints (numbered) concern: (1) Signal (S) recognition by the SRP54 M domain. (2) Elongation arrest by the Alu domain. (3) Early TC formation with the SR at the proximal site of SRP RNA. (4) Translocon mediated displacement of the TC and docking of the RNC on the translocon. (5) Signal release into the translocon and binding of the TC on the distal site. (5a) GTP hydrolysis within SRβ and SRX dissociation. (6) Activation of GTP-hydrolysis within the TC (T* for activated GTPs) by the 5f-loop. The phosphorylated carboxyl-terminus of SRP72 (denoted with P) is in proximity to the activation site. The tail enhances translocation and is cleaved during apoptosis and relevant in autoimmune diseases.

with the SRX domain of SRα is well known and its GTPase switch cycle has been described (Jadhav et al., 2015b). The problem starts with the description of the corresponding GTPase-activating protein (GAP) and GEF functions and respective checkpoints for this third GTPase. SRX apparently acts as co-GAP, but is not able to stimulate hydrolysis by itself (Schlenker et al., 2006). Previous data suggested the SRP-RNC to interact only with SRβ-GDP and the translocon with SRβ-GTP (Fulga et al., 2001). In cryo-EM reconstructions, SRαβ was modeled onto the distal site of SRP RNA (Halic et al., 2006b), which might therefore act as a general activating site for all three GTPases. However, in the recent mammalian pre-handover structure, the SRβ/SRX complex is shifted and has been modeled in vicinity to the TC, next to the SRP68-RBD (Kobayashi et al., 2018), which due to limited resolution could also correspond to the still unresolved SRP68 central TPR regions. In a scenario integrating all data, the regulation of the SRβ GTPase apparently constitutes an additional checkpoint (5a) necessary for signal release (Figure 7). Finally, the GEF function in yeast has been attributed to the Sec61 translocon, but no high-resolution structure of a GAP or GEF complex is available to shed light on SRβ regulation.

Taken together, despite the universal conservation of SRP, evolution has created a variety of structural and functional adaptations of SRP that need to be elucidated in detail. Extending the studies to more organisms and phyla

in the future is necessary to create a precise roadmap of the molecular mechanisms of SRP.

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