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SmpB as the handyman of tmRNA during trans-translation

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In the recent years, a wealth of genetic, biochemical and structural data focusing on various steps of bacterial trans-translation has been reported. The early events, from stalled ribosome recognition, pre-accommodation to translocation have been recently investigated in great details. In comparison, the later events including 'elongation-termination' onto tmRNA reading frame and ribosome recycling are much less known. What follows is a summary of those data together with our personal view of the dynamics of trans-translation, with emphasis to the pivotal and leading roles that the SmpB protein has, being the essential conductor during the whole process.

The Why and How of Trans-Translation in Bacteria

In bacteria, ribosome stalling at the 3'-end of mRNAs lacking termination codons is a serious issue for cell survival. Arrested ribosomes cannot be recycled back to new rounds of translation since they are unable to terminate protein synthesis using release factors. If not rescued, most ribosomes would become inactive very fast during bacterial growth. In all eubacteria and some organelles, there is a specialized quality-control mechanism for rescuing ribosomes arrested during translation performed by the 'tmRNA-SmpB' (transfer-messenger RNA-Small protein B) ribonucleoprotein complex.[1] In a reaction known as trans-translation, bi-functional tmRNA possessing both a tRNA and a mRNA domain, in complex with SmpB, recognizes the stalled ribosomes and adds an internally encoded peptide tag to the C terminus of nascent polypeptides for destruction by cellular proteases. The ribosomes dissociate from tmRNA at its internal stop codon and are recycled back to new rounds of translation.[2] Ribosome recycling is its primary function whereas protein tagging and elimination is being accessory. Recent studies suggest a broader physiological role for trans-translation in monitoring protein folding and co-translational events.[3]

tmRNA safeguard, activation and shuttling. In all species encoding and expressing tmRNA, it contains a partial tRNA domain (TLD for tRNA-like domain) aminoacylated at its 3'-end by alanyl-tRNA synthetase, but restricted to a tRNA acceptor branch (acceptor stem and T stem-loop) containing

modified nucleosides, as for canonical tRNAs,[4] as well as several pseudoknots and helices (**Fig. 1A** and reviewed in refs. 5 and 6). The small basic SmpB protein is essential for trans-translation, enhances tmRNA aminoacylation and prevents its degradation[7,8] binds tmRNA[9] and the stalled ribosomes[10] and is required for loading Ala-tmRNA, in complex with EF-Tu[11] onto the empty (or partially empty) A site of the stalled ribosomes. During the past 10 y, a large body of biochemical, genetic and structural data has accumulated on bacterial trans-translation, converging to a reasonably well experimentally supported scenario. Alanyl-tmRNA bound to SmpB and EF-Tu enters the A-site of a stalled 70S ribosome having a peptidyl-tRNA in the P-site. tmRNA tRNA domain is near the peptidyl-transfer active site and SmpB is at the decoding center, mimicking functionally a 'codon-anticodon' interaction and structurally a tRNA anticodon branch corresponding to the anticodon and D stems of a tRNA.[12,13] The nascent polypeptide gets transpeptidated to the alanine at tmRNA 3'-end and the tag reading frame is inserted within the ribosome decoding center. Translation continues to a canonical termination codon onto tmRNA, releasing the aborted nascent protein for destruction by cellular proteases.

Handy SmpB. The SmpB protein is an essential component for trans-translation in vivo, responsible of a variety of actions in the course of the mechanism. It binds with high affinity to tmRNA in vivo,[9] protects it from degradations in the cells (3-fold shorter half-life of tmRNA in the Δsmpb cells compared with wild-type cells, Hallier et al.[18]) and enhances its alanylation efficiency.[14] Moreover, SmpB is required for stable association of tmRNA with the stalled ribosomes in vivo,[9] tightly interacts with the stalled ribosomes in vivo,[10] presumably to facilitate the recruitment of ala-tmRNA into a vacant A site. SmpB 3-dimensional structure forms an oligonucleotide-binding (OB) fold made of 6 antiparallel β-strands that create a closed β-barrel exposing 2 conserved RNA-binding domains on opposite sides (**Fig. 1B** and reviewed in refs. 15–17). Similar OB-folds were detected on other RNA-binding proteins involved in translation, including the initiation factor IF1.[15] Of the 160 amino acids in *E. coli* SmpB, the C-terminal 30 residues comprise a tail that, while unstructured in solution and not observable in NMR or crystal structures, performs an essential function in trans-translation. SmpB is mandatory at each step of the process of trans-translation.

Key Questions about the Mechanism: Some Recently Answered, Some Yet Unanswered

Such a peculiar quality control system calls on to provocative questions about the mechanisms. How the stalled ribosomes, *vs.* the active and translating ones, are discriminated? What is the accurate sequence of molecular events leading to trans-translation? How 'tmRNA-SmpB', that is six times larger than the tRNAs, can make its way through the stalled ribosomes? How the 'SmpB-tmRNA' duplex progress from the A to the P sites and from the P to the E sites? What are the functional roles of the pseudoknots? Preventing degradation, providing recognition motives for the ribosome? Is their dynamics of opening and closing required for reading frame selection and progression within the ribosomes? What happens to the tRNA domain of tmRNA once released from the E site? What is the exact functional role of SmpB C-terminal tail?

Tentative models of the detailed mechanism have been proposed in the past years, trying to answer these questions. Based on the latest biochemical, genetic and structural studies, this review aims at presenting those proposals, together with our personal views of the dynamics of bacterial trans-translation.

Roles of SmpB for recruiting tmRNA to the stalled ribosomes. Specific binding of SmpB into an empty decoding site on the small subunit is required for tmRNA to be recognized and correctly positioned into a stalled ribosome. While SmpB is required for ala-tmRNA to bind the stalled ribosomes, the opposite is not true: SmpB can bind tightly to 70S ribosomes in the absence of tmRNA, either in vitro or in vivo.[18,19] This raises the question of the chronology of the early events leading to trans-translation initiation. Is a pre-formed

quaternary complex made of 'ala-tmRNA-SmpB-EF-Tu·GTP' indispensable to trigger trans-translation or does the pre-binding of SmpB pave the way for ala-tmRNA to engage? By monitoring the cellular location and expression of endogenous SmpB, we previously reported that it is associated with 70S ribosomes and that this pre-bound SmpB can trigger the recruitment of SmpB-free tmRNA and initiate trans-translation in vitro.[18] On the other hand, the intracellular concentrations of tmRNA and SmpB being roughly similar,[20] the formation of a 1:1 complex between them would be in contradiction with the pre-binding of SmpB to the stalled ribosomes. Interaction of free SmpB with ribosomes is salt sensitive in vivo and therefore could be dependent upon the low stringency conditions used in the purification buffers, while its high binding affinity to tmRNA is unquestionable.[21] To our point of view, because SmpB was found in vivo bound to the ribosome and because the intracellular ionic environment can vary, we cannot rule out that this original route for initiating trans-translation might be used, at least under specific cellular conditions.[18] In any case, SmpB acts as a cellular sentinel onto the stalled ribosomes pinpointing those to be rescued.

Stalled ribosomes detection and scanning for an empty A-site. Trans-translation was initially shown to occur on ribosomes stalled at the very 3'-end of incomplete mRNAs lacking a stop codon and therefore carrying an almost or totally empty decoding site.[2] These non stop mRNAs can result from (1) mutations causing the lack of in-*frame* termination codons (2) a premature termination of transcription before the termination codon is reached[22] (3) mRNA cleavages by nucleases.[23] This situation occurs also when the stop codon is bypassed by unwanted translational read-through caused by nonsense suppressor tRNAs,[24] by the presence of miscoding antibiotics[25] or by aberrant frameshifts.[26] More intriguingly, trans-translation can also be triggered on intact full-length mRNAs when some internal sites are paused into the ribosomal A-site. These sites can be (1) clusters of rare codons,[27] (2) weak termination codons.[28-30] They can also be the result of a flawed co-translational process, such as wrong protein folding or secretion; then, trans-translation is necessary to relieve the subsequent translational arrests, whatever the codon context.[3] The mechanism by which trans-translation is activated on these full-length mRNAs has been elusive until the discovery of the role played by the bacterial toxin RelE in inhibiting protein synthesis under nutrient deprivation conditions. Indeed, during bacterial amino acid starvation, ribosomes are stalled by the binding of deacylated tRNAs to their A sites. The stringent factor RelA then binds to blocked ribosomes and catalyzes synthesis of (p)ppGpp, a secondary messenger that induces the stringent response.[31] This situation is cooperating-competing with RelBE and tmRNA.[32] RelE is part of the *relBE* toxin/antitoxin system in which it plays the role of the toxin while RelB is the unstable antidote bound to the latter. The concentration in RelB is decreased by the arrest of protein synthesis, leading to the delivery of the stable RelE toxin that in turns cleaves mRNA codons between the second and third nucleotides in the A site of translating ribosomes.[33,34] This situation leads to non-stop mRNAs, the typical substrates of trans-translation (see above). A-site mRNA cleavage by bacterial toxins during the pausing of translating

ribosomes seemed therefore to be the missing link explaining the way trans-translation occurs on full-length mRNAs. However, a few months after this striking discovery, it was shown that the A-site specific cleavage into paused ribosomes could also occur in the absence of RelE or other bacterial toxins, and do not require tmRNA, SmpB, ribonucleases R, E, G and III or (p)ppGpp.[1,26] Despite extensive studies, this novel endonucleolytic activity has not yet been attributed, suggesting that the ribosome itself might participate to the process, in a way that is still unknown. Last but not least, trans-translation can, in some cases, be triggered without a previous endonucleolytic activity cleaving the mRNA into the A-site. Indeed, the translation of bacterial mRNA begins while the nascent transcript is being synthesized and the active RNA polymerase partly protects the mRNA downstream the working ribosomes. When translation pauses, the ongoing RNA polymerase synthesizing the mRNA keeps moving and part of the mRNA extending downstream the leading edge of the ribosome becomes exposed to the cellular endonucleases and to 3'–5' exonucleases. This way, only the nucleotides between the head and the shoulder of the small subunit are maintained protected against rapid degradation. Between the P-site and the 3' edge, this region corresponds to ~15 nucleotides.[35] Accordingly, ribosomes stalled at mRNAs are targets for trans-translation only if the extension does not exceed 15 nucleotides downstream the A-site. Over this length, the rates of trans-peptidation decrease up to zero.[36] Therefore, in this "edge model," the 'tmRNA-SmpB' complex competes with the nucleotides remaining inserted into the mRNA path of the ribosome that may be transiently detached from the A-site because of weak interactions with the mRNA channel.[37] Altogether, these situations lead to high frequency of trans-translation, of about 1 in 250 translation events while ~700 tmRNA are present per cell, corresponding to one tmRNA for 10 to 20 ribosomes.[38] This prevalence of trans-translation is not increased in cells overexpressing tmRNA and SmpB, arguing for a tightly regulated entry of the 'tmRNA-SmpB' complex into the A-sites of the stalled ribosomes.

Signatures of ribosome stalling; SmpB interactions with the ribosomal active sites. Finally, despite the wide range of situations described above, ribosome pausing or idling is the common rule governing the process of trans-translation. Because of the strong competition between trans-translation and termination or elongation (more particularly in the "edge model" situation), an accurate identification of these problematic ribosomes by the 'tmRNA-SmpB' complex is necessary and needs very specific signatures to discriminate them from the active ones. A vacant A-site is the most obvious signal making a ribosome a good substrate for SmpB and therefore ala-tmRNA. So how to detect ribosomes stalled at the 3' edge and still carrying a codon into the A-site? When the ribosomes stall at or very near the 3'-end of the mRNA it has been suggested that the opening of the mRNA channel may facilitate the subsequent identification and engagement of 'tmRNA-SmpB', by providing a positive mechanism of identification.[1] Although this situation can occur on empty A-site ribosomes, it is unlikely that ribosomes will distinguish by themselves long mRNAs from the short ones paused at the 3'edge. Therefore we anticipate that in the "edge model," opening the
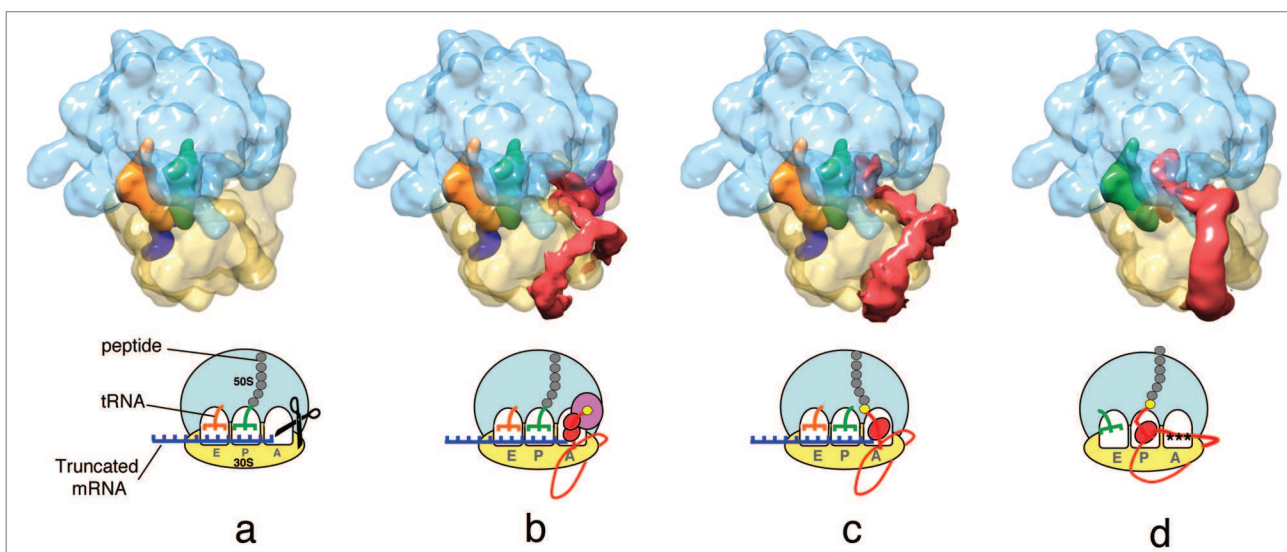
Figure 2. Cryo-EM maps of the currently solved functional complexes. Stalled ribosomes (a), pre-accommodation of the 'tmRNA-SmpB' complex (b), accommodation (c) and translocation (d). The density attributable to 'tmRNA-SmpB' is in red, the 50S subunit is blue, the 30S subunit is yellow, and the P-site and E-site tRNAs are depicted in green and orange, respectively, and the problematic mRNA is purple. The semi-transparent ribosomal subunits emphasize the relative positions of the 'tmRNA-SmpB' complex, P-site and E-site tRNAs in the 3 active sites of the ribosome. For clarity, each of the 4 steps was schematized below the cryo-EM structures.

mRNA channel may rather be the task of SmpB competing with the remaining nucleotides to pave the way for tmRNA to enter.

The SmpB protein has a β-barrel core structure and a C-terminal tail that gains structure within the ribosome and its deletion abolishes tagging.[39] Based on cryo-EM reconstructions[40] and directed hydroxyl radical probing,[41] into the A-site, SmpB mimics a 'codon-anticodon' pairing. Accordingly, chemical probing assays and NMR[42] have shown that SmpB protects nucleotides G530, A1492 and A1493 of the 16S rRNA from being modified. These key conserved nucleotides from the decoding site of the small subunit undergo substantial rearrangements in response to the pairing of cognate codons and anticodons,[43] binding of IF1,[44] binding of antibiotics[43] or, to a less extent, recognition of a stop codon by release factors RF1 or RF2.[45-47] However, in spite of the observed reactivity changes, mutations at these positions do not reduce SmpB binding to the decoding site[42] or reduce the rate of peptidyl-transfer onto tmRNA (A. Buskirk, personal communication). Therefore, other contacts between SmpB and the decoding site that are different from those induced during translation elongation, are likely to take place with the surrounding nucleotides of the 16S RNA since positively charged SmpB has potential for interacting with negatively charged rRNA. X-ray structures will be necessary to provide detailed insight into the loading of the 'tmRNA-SmpB' complex within a stalled ribosome.

The C-terminal tail of SmpB plays a crucial role during trans-translation, which led to an extensive questioning about its function during the early steps of the process. First of all it does not contribute to the binding of the protein to the ribosome, nor to the GTP hydrolysis by EF-Tu as demonstrated by testing various mutated or tail-truncated proteins.[10,39,48,49] Therefore, its function is crucial for the events that are after the initial association with the ribosome but before transpeptidation.[39] It is suggested that

the tail, which is disordered in solution, forms an α-helix that interacts with the mRNA path downstream from the A-site when entering the ribosome.[41,49] This interaction occurs at pre-accommodation and it triggers the accommodation of tmRNA into the A-site after GTP hydrolysis by EF-Tu, compensating for the lack of a codon during the entering of tmRNA while the upper half of tRNA is mimicked by the tRNA domain (TLD).[50] The positioning of SmpB into the decoding site is consistent with the crystal structures of the protein bound to the TLD.[12,13] Overall, the 'RNA-protein' complex mimics the L-shaped conformation of a canonical tRNA. tmRNA terminus corresponds to the acceptor and T arms of the upper part of a tRNA while the positioning of SmpB mimics the anticodon and D stem-loops. The super-imposition of these structures on the A-site of a 70S ribosome orientates the C-terminal domain of SmpB to the decoding site while the β7 strand corresponds to the anticodon loop. These observations confirm the structural mimicry of canonical tRNAs by a 'SmpB-tmRNA' complex while the H2 linker helix mimics a tRNA variable arm (**Fig. 1C**).

Trans-translation dynamics under the eye of a microscope. *Ribosome recognition: the pre-accommodation step.* The first views of a complete 'tmRNA-SmpB' complex loaded onto a stalled ribosome were determined by cryo-electron microscopy (cryo-EM, **Fig. 2**). The pre-accommodation step was captured by using kirromycin antibiotic that allows GTP hydrolysis by EF-Tu but stalls the 'EF-Tu•GDP' complex with 'tmRNA-SmpB' on the ribosome before accommodation into the peptidyl-transferase site. In an initial model, the TLD was placed into the density to interact with the GTPase-associated center (GAC) in the 50S subunit and with protein S12 in the 30S subunit, in a way similar to that of tRNA during the elongation cycle. In this model, SmpB interacts with the elbow and the lower portion of TLD

and bridges the latter to the 50S subunit. tmRNA helix 2 is redirected along the 30S subunit beak, where pseudoknots PK1 to PK4 form an arc that direct the internal open reading frame close to the entrance of the mRNA channel in the 30S subunit.[51] In a second map with improved resolution (**Fig. 2b**) and thanks to the docking of the crystal structure of the tRNA domain of tmRNA in complex to SmpB[12] we established the occurrence of 2 SmpB molecules in the complex, one interacting with the 50S subunit at the GTPase-associated center (GAC) near the site where it was previously found (SmpB-1), the other (SmpB-2) to the 30S subunit close to the decoding site.[52] This result agrees with the distribution of the protections induced by the SmpB protein onto tmRNA as well as with biochemical data suggesting that the protein binds to the opposite sites of helix H2 from tmRNA.[19,53]

Why needing a second SmpB? During standard translation elongation, codon recognition leads to a series of conformational changes that position EF-Tu for GTP hydrolysis.[54] Among these changes, a domain closure of the 30S subunit occurs together with distortions of the tRNA backbone within the anticodon and D stems that are required to simultaneously bind the mRNA codon and EF-Tu.[55] The interactions of EF-Tu with the distorted tRNA and the ribosome activate GTP hydrolysis and the subsequent dissociation of the protein triggers the accommodation of the tRNA into the A-site. At this step, "proofreading" allows the disengagement of tRNAs interacting weakly (i.e., near-cognate tRNAs) with the decoding center.[56] SmpB compensates for the lack of 'codon-anticodon' recognition, therefore being essential during the initial selection of the stalled ribosomes to rescue. "Non-cognate" proofreading is probably unnecessary in this step. An hypothesis would be that SmpB-1 interacting with the 50S forms close contacts with the GAC including the nucleotides from the 23S rRNA which interact with the D loop of tRNAs during canonical translation. Thus, this interaction may be required to transfer the "decoding" signal to EF-Tu and to facilitate subsequent GTPase (Guanosine TriPhosphatase) activation.[52] The simultaneous binding of 2 SmpB proteins during pre-accommodation is controversial, mainly because of the '1:1' ratio of SmpB and tmRNA measured within the cells.[20] However, if we consider that SmpB-1 leaves the stalled ribosome as soon as the TLD accommodates into the A-site (see below), the presence of 2 molecules of SmpB during pre-accommodation is compatible with the estimated '1:1' ratio of the 'tmRNA-SmpB' complex in vivo. Indeed, trans-translation is a multi-step process that can be roughly divided into three main phases: (1) pre-accommodation and accommodation of the TLD into a vacant A-site, (2) template swapping and selection of the reading frame on tmRNA (3) elongation on the 9–35 (depending on the species) internally encoded codons of tmRNA open reading frame and termination. The presence of a '2:1' SmpB/tmRNA ratio during the accelerated pre-accommodated state is insignificant compared with the remaining steps of the process for which only one SmpB accompanies tmRNA during its transit through the stalled ribosomes,[57] including accommodation, elongation onto tmRNA ORF and termination. Further structural studies will be required to provide a clearer answer to that controversy regarding the number and dynamics of SmpB molecules during pre-accommodation.

Swinging toward the accommodation step. Structural information about the post-accommodated state of the 'tmRNA-SmpB' complex comes from cryo-EM studies. In a first study using a truncated version of tmRNA (named PKF for pseudoknot-free tmRNA) we showed that accommodation leads to the disappearance of SmpB-1 from the large subunit while SmpB-2 remains bound into the decoding site.[58] The signal triggering SmpB-1 departure is explained by the steric clash that the protein would provoke with the P-site tRNA after the release of EF-Tu·GDP and swinging of the TLD into the A-site. At the same time, an independent study by Lindahl's group has reported the accommodation step in the presence of full-length tmRNA.[59] Despite the high heterogeneity of the samples (as we previously observed in refs. 52 and 58) and the lack of deacylated tRNA into the P-site, the structure of the complex at 15 Å resolution confirms the absence of SmpB-1 and the movements of the 'TLD-SmpB' complex as do native aminoacyl-tRNAs during canonical translation. It also reveals that the large 'arc-shaped' density made by the pseudoknots and internal ORF of tmRNA remains folded and highly structured around the beak of the 30S subunit (**Fig. 2c**). We recently revisited 'tmRNA-SmpB' accommodation in a novel structure refined at 13 Å resolution.[60] From the original cryo-EM density map we constructed an atomic model that was optimized by 'molecular dynamic flexible fitting'. As expected, the TLD contacts with the large ribosomal subunit resemble those of the accommodated canonical tRNAs while the lack of a D-stem is compensated for by SmpB. This positioning is instrumental in realigning H2 (the helix connecting the TLD to the arc-like rest of tmRNA) toward the large subunit, in which it makes extended contacts with protein L11. Compared with the pre-accommodated step, SmpB follows the swing of the TLD by a slight rotation of about 30° but stays at the same place into the decoding site, still mimicking an anticodon stem-loop. The ring of pseudoknots does not undergo a large movement and still wraps around the beak of the small subunit.

P-site translocation: tRNA anticodon mimicry and tmRNA frame selection. After transpeptidation, the TLD has to move to the P-site while the internal open reading frame of tmRNA engages into the mRNA path. Until recently, the way such a huge molecule (six times larger than a tRNA when in complex with SmpB), with stable secondary and tertiary structure moves trough the ribosome was still a central question for understanding the mechanism of tmRNA function.[61] Recently, our group and that of J. Frank addressed the conformational changes in the 'tmRNA-SmpB' complex when it moves through the ribosome (**Fig. 2d**), especially those required for the transition from the A- to the P-site.[60,62] The translocation step implies dynamical motions of tmRNA-SmpB but also of the ribosome to (a) allow the complex to cross the hurdles between the A and P-sites (b) swap the mRNA templates (c) select the correct resume codon and maintain the reading frame on tmRNA.

Crossing the hurdles. During ribosomal translocation, ribosomal subunits need to move relative to each other, underscoring the dynamic nature of the ribosome.[63] This movement leads to a ratchet-like rotation of the 30S subunit relative to the 50S
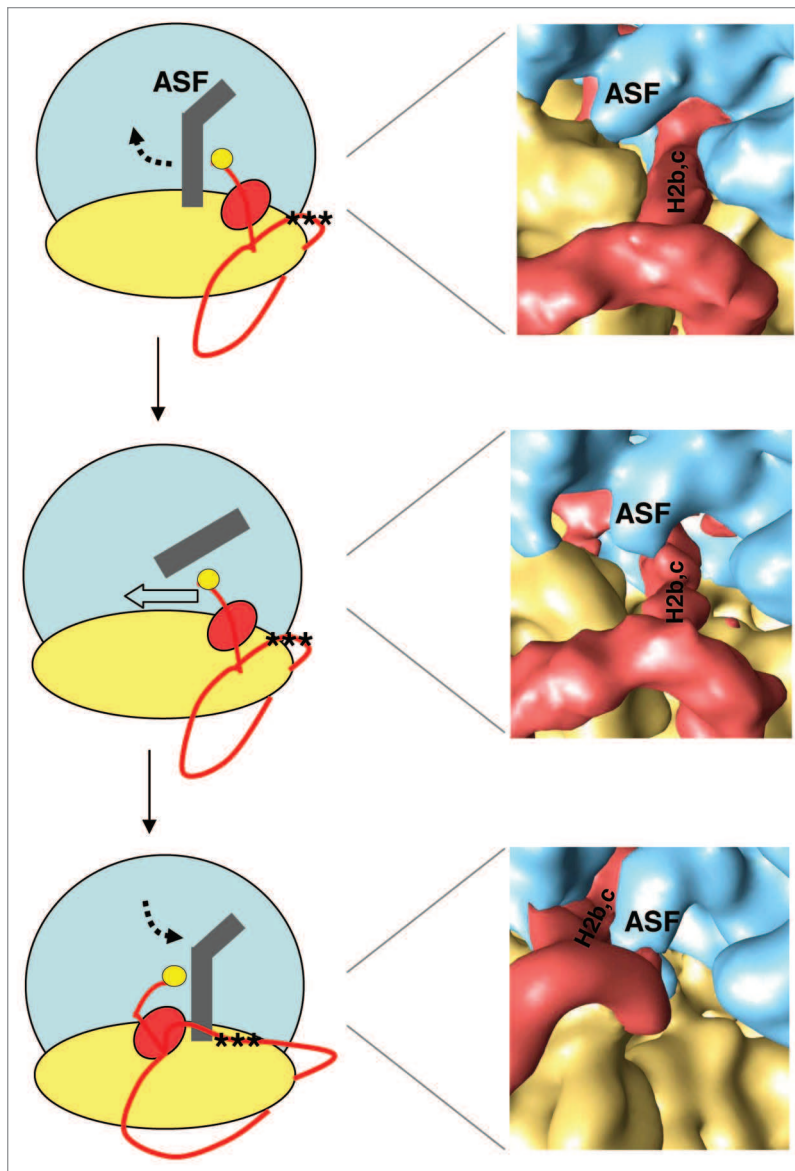
Figure 3. A-site finger (ASF, in gray) motion (see ref. 81 for more details) as the 'tmRNA-SmpB' complex (red) translocates to the P site. (Left) Schematic views of how the 'tmRNA-SmpB' complex crosses the ASF before, during and after translocation. (Close-up) Cryo-EM maps of stalled 70S ribosomes with 'tmRNA-SmpB' accommodated (top), the 70S ribosome in the ratcheted state prior to 'tmRNA-SmpB' translocation (middle), and the 70S ribosome with 'tmRNA-SmpB' translocated into the P site (bottom). During translation, the movement of the ASF is illustrated by a broken arrow and the position of the resume codon corresponds to the 3 black stars.

the ratchet, the axis of rotation has been located in the vicinity of the central bridge 3 and consequently, only the bridges located at the extremities of the 2 subunits (B1a-b; B7b and B8) are disrupted or rearranged during rotation.[67] Among them, we have shown that following ratcheting, disruption of the A-site finger or ASF, part of bridge B1a, allows the large 'tmRNA-SmpB' complex to make its way through the narrow pathway of the canonical tRNAs.[60] Indeed, this disruption favors the passage of H2 to the other side of the bridge (**Fig. 3**). As a consequence, pseudoknot PK1 moves at the entrance of the ASF gate, where H2 was previously positioned, while the 3'-end of PK4 also follows the dynamics of H2. On the other hand, the 5'-end of PK4, together with H5/PK2/PK3 stay immobile, triggering the stretch of the arc-shaped ring of PKs and the unfolding of the internal ORF.

mRNA swapping. According to the general process of trans-translation, the stalled ribosome has to rapidly switch RNA templates to set tmRNA in the mRNA mode. During stalling, the truncated mRNA is stabilized into its path mainly through 'codon:anticodon' interaction with the peptidyl-tRNA. 'tmRNA-SmpB' recruitment is followed by rapid trans-peptidation that destabilizes the P site-bound tRNA, which in turn dissociates the mRNA from the ribosome.[68] This early release of the stalled mRNA has been recently confirmed by cryo-EM. Indeed, the translocation of tmRNA-SmpB to the P-site is accompanied by the disappearance of an extra-density recovered between the head and the back of the small subunit platform, where the Shine-Dalgarno interaction between the stalled mRNA and the 16S RNA occurs.[60] Once it is released, the flawed mRNA has to be rapidly degraded to avoid a new round of flawed translation to start. Noteworthy, trans-translation promotes the degradation of non-stop mRNAs that cause ribosome stalling.[23] Among all the RNases potentially involved, the 3' to 5' exoRiboNuclease R is the key enzyme in tmRNA-dependent non-stop mRNA decay, in a process that requires active trans-translation of the defective mRNA.[69]

Resume codon selection within tmRNA. As the tmRNA-SmpB complex transits to the P-site, the ribosome has to select the correct reading frame on tmRNA. A wealth of biochemical and genetic data have suggested that interactions between SmpB and key nucleotides lying upstream of the resume codon are instrumental in setting the new frame.[21,70-74] At this step, the position of SmpB has been mapped by the sites of cleavages induced by hydroxyl radical probing of Fe(II) tethered SmpB mutants. Contrary to the A-site position that protruded into the mRNA path toward the downstream tunnel, they localize almost exclusively around the region of the P-site canonical codon-anticodon interaction.[41] Strikingly, this localization allows SmpB to have direct

subunit in the direction of the mRNA movement.[64] Ratcheting involves a reversible ~8° inter-subunit rotation and a nearly orthogonal rotation of the head domain of the small subunit.[65] The interface between the two subunits rearranges along the ratcheting pathway and imposes stringent limitations on the accessible pathways for tRNAs. This rotation triggers independent movement of the two extremities of tRNAs, leading to the A/P and P/E hybrid states, necessary for the subsequent translocation of tRNAs. The two subunits of the ribosome are linked together by at least 12 inter-subunit bridges.[66] During

contacts with the area comprising the 5 nucleotides upstream of the resume codon (**Fig. 4**), which precisely places the latter at the ribosomal A-site.[60,62]

E-site translocation and ribosome exit. The model presented here lends support to a mechanism in which the next transition of tmRNA-SmpB from the P- to the E-site would follow the same dynamics (**Fig. 5**). In line with the first translocation, the movement of the TLD to the E-site is likely to drag H2 across bridge B1b, made by the flexible interactions between L5 and S13 [64,75] and the crossing of the following module made of PK1 over bridge B1a. Accordingly, an isolated tmRNA-ribosomal complex blocked with tmRNA in the E-site was recently analyzed using chemical probing. The model resulting from this study suggests a positioning of PK1 and H2 nearby the E-site.[76] The dynamics of the other modules are more elusive and mainly depend on when helix H5 will come out of the ribosome and unwound to be correctly translated into the mRNA path.

Concluding Remarks

'tmRNA-SmpB' movements in the context of the polysomes. During translation, a cluster of ribosomes bind to a single mRNA, giving birth to an active polyribosome or polysome. When the first ribosome stalls at the 3'-end of a no-go mRNA, it leads to a traffic jam of all the following polysome. Therefore, after salvage of this first ribosome by tmRNA-SmpB, the polysome moves up on the flawed mRNA that must not be subjected to re-initiation at the risk of leading to a translation/trans-translation vicious circle. Therefore, a competition occurs between the polysome moving forward on the mRNA and degradation by RNase R. Another possibility is that the 5'-end of the flawed mRNA is degraded as trans-translation proceeds, preventing re-initiation. Further analysis of stalled polysomes will be needed to answer this question.

Alternative ways of resolving stalled ribosomes. Transtranslation is not essential in most bacteria, suggesting that there are alternative roads for rescuing the ribosomes that stall on problematic mRNAs. One possibility might be peptidyl-tRNA drop-off promoted by translation factors EF-G, RF3 and RRF, with subsequent hydrolysis of the peptidyl-tRNA by the peptidyl-tRNA hydrolase (Pth). Overexpressing RF3, RRF and the Pth, however, does not stimulate peptidyl-tRNA production,[77] arguing against the peptidyl-tRNA drop-off hypothesis. Recently, YhdL (or ArfA for alternative ribosome-rescue factor) was identified as an essential protein for *E. coli* viability in the absence of tmRNA, ArfA taking over the role of the 'tmRNA-SmpB' complex.[78] Interestingly, ArfA lacks the catalytic residues of the peptide release factors, therefore requiring an additional ligand able to hydrolyze the bond between the tRNA and the nascent peptide, possibly the Pth. In addition to, the ArfA system may be required during different physiological conditions than transtranslation or, alternatively, when the 'tmRNA-SmpB' quality-control mechanism is outreached.

Trans-translation as an antimicrobial drug target. In some bacteria (e.g., *S. typhimurium*, *Y. pseudotuberculosis*) responsible of human and/or animal diseases, functional links were
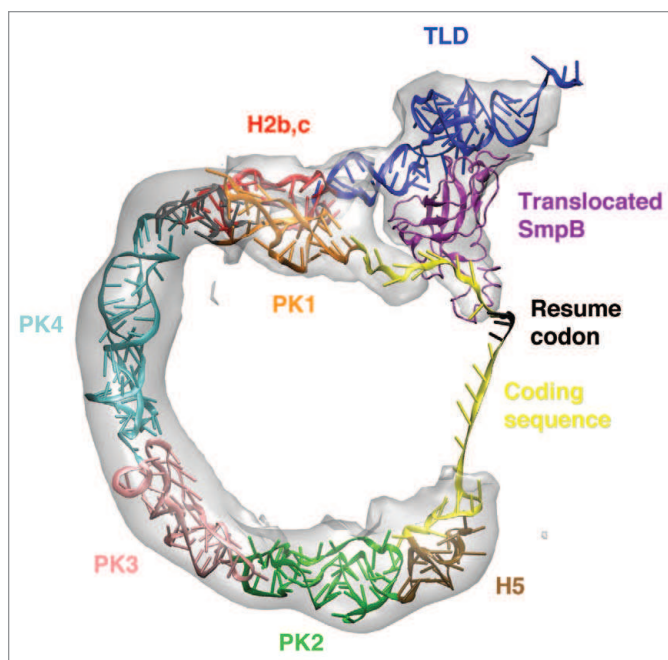

Figure 4. Cryo-EM structure of a translocated 'tmRNA-SmpB' complex at 13.5 Å resolution (Weis et al. 2010), highlighting the contacts between SmpB and the conserved nucleotides upstream the resume codon. The electron density corresponding to the 'tmRNA-SmpB' complex is in gray and the model reconstructed and fitted within the electronic density follows the ribose-phosphate backbone of the RNA and the ribbon backbone of the protein. The color code is as in **Figure 1**.

substantiated between trans-translation and virulence. In specific infections such as gonorrhoea, bacteria cannot survive without trans-translation. Also, trans-translation can contribute to cell viability in the presence of protein synthesis inhibitors,[79] because these molecules induce miscoding events or extended stalling during translation. The 'tmRNA-SmpB' duplex allows bacteria to recycle the ribosomes stalled by the antibiotics, allowing reusing them onto intact messages, therefore increasing cell viability. Trans-translation also impacts the activity of antibacterial drugs that inhibit cell wall synthesis, probably because these drugs induce an overall stress to the bacteria that will be suppressed more efficiently when trans-translation is active.[80] Altogether, these data suggest that either tmRNA, SmpB or the 'tmRNA-SmpB' complex, all structures known at the atomic levels, are promising targets for developing novel antibiotics. Molecules that could bind tmRNA, SmpB or both might interfere with their loading onto the ribosomes to rescue, potentiating the action of the existing drugs and allowing decreasing their active concentrations to limit their side effects. Since trans-translation is missing in the eukaryotes, these drugs should have reduced side effects on the cells and metabolisms of the patient.
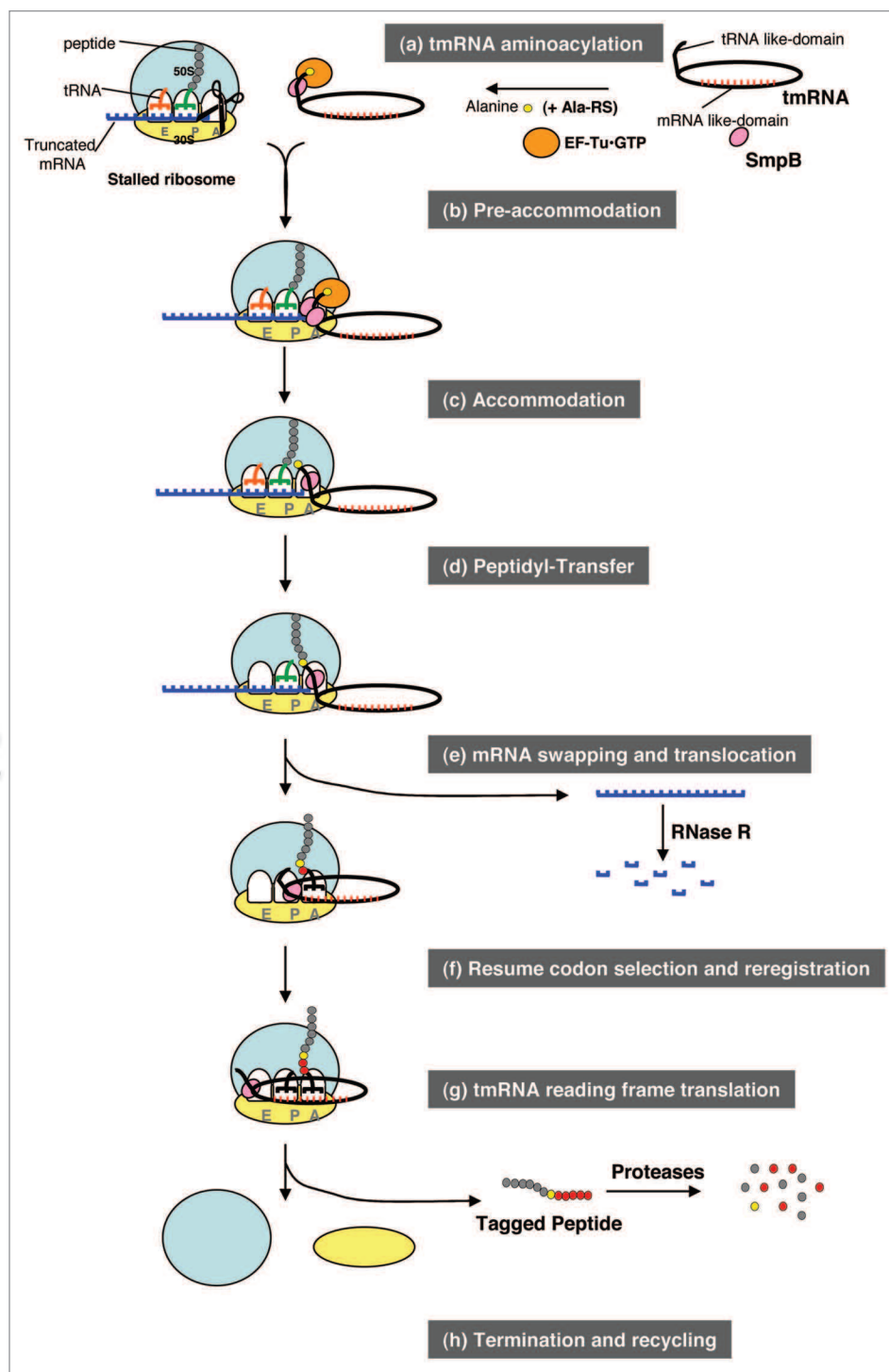
Figure 5. Working model of the mechanism of trans-translation based on a wealth of genetic, biochemical and structural data collected over the past 15 y. (a) Ribosomes stalled on a problematic message, e.g., a truncated mRNA (blue, with a black scissor), contain an E-site tRNA (orange) and a peptidyl-tRNA within the P site (green). Alanylated (yellow marble) tmRNA in complex with EF-Tu·GTP (orange circle) and with SmpB (pink) recognize an empty or partially empty A site, with SmpB mimicking a tRNA anticodon branch contacting the small ribosomal subunit. (b) During pre-accommodation, a second, transient, SmpB molecule contacts the GTPase center of the large ribosomal subunit, presumably facilitating GTP hydrolysis after binding of the first SmpB into the decoding site. (c) Accommodation occurs as for canonical tRNAs during translation, together with the departure of the second SmpB molecule. (d) The incomplete peptide is transferred from the P site tRNA to the alanine located at tmRNA 3'-end. (e) The problematic mRNA is ejected and rapidly degraded by RNases (RNA quality-control system) and 'tmRNA-SmpB' translocation from the A to the P site takes place. (f) tmRNA internal resume codon is placed at the ribosomal decoding site with the help of SmpB that provides specific contacts with nucleotides upstream of the tag-encoding sequence. (g) Translation of tmRNA internal reading frame continues and ends at a canonical termination codon, releasing the aborted nascent protein for destruction by cellular proteases. (h) The stalled ribosomes are recycled back to new rounds of translation onto intact mRNAs. The remaining color code is as in **Figure 2**.

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