REVIEW ARTICLE

The bacterial translation stress response

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Abstract

Throughout their life, bacteria need to sense and respond to environmental stress. Thus, such stress responses can require dramatic cellular reprogramming, both at the transcriptional as well as the translational level. This review focuses on the protein factors that interact with the bacterial translational apparatus to respond to and cope with different types of environmental stress. For example, the stringent factor RelA interacts with the ribosome to generate ppGpp under nutrient deprivation, whereas a variety of factors have been identified that bind to the ribosome under unfavorable growth conditions to shutdown (RelE, pY, RMF, HPF and EttA) or re-program (MazF, EF4 and BipA) translation. Additional factors have been identified that rescue ribosomes stalled due to stress-induced mRNA truncation (tmRNA, ArfA, ArfB), translation of unfavorable protein sequences (EF-P), heat shock-induced subunit dissociation (Hsp15), or antibiotic inhibition (TetM, FusB). Understanding the mechanism of how the bacterial cell responds to stress will not only provide fundamental insight into translation regulation, but will also be an important step to identifying new targets for the development of novel antimicrobial agents.

Introduction

The ribosome is the protein-synthesizing machine in the cell and therefore plays a major role in determining the overall gene expression profile of the cell. There is ever-increasing evidence that numerous protein factors interact with the ribosome to regulate protein synthesis and modulate the expression profile of the cell in response to different environmental stresses. This review aims to provide an overview of the variety of diverse protein factors that interact with the ribosome and highlight how they accomplish their function under such stress conditions. Table 1 provides a summary of the ribosome-associated factors addressed in this review, with a brief description of their mechanism of action. However, to fully appreciate how these factors alter ribosome function, a basic understanding of the ribosome itself and the process of protein synthesis in bacteria is required.

The bacterial 70S ribosome is a ribonucleoprotein particle composed of two subunits, a small 30S and a large 50S subunit (Wilson & Nierhaus, 2005). The *Escherichia coli* 30S subunit is composed of a 16S ribosomal RNA (rRNA) and 21 ribosomal proteins (S1–S21), whereas the 50S subunit comprises a 5S and 23S rRNA as well as 33 ribosomal proteins (L1–L35). The bacterial 70S ribosome provides a platform upon which aminoacyl-tRNAs (aa-tRNAs) can bind and their respective amino acids can be linked together to form a polypeptide chain. The main role of the 30S subunit is to ensure that the mRNA is correctly positioned to maintain the reading frame of the protein and to ensure that the tRNAs bearing the appropriate amino acids are paired with the correct codons of the mRNA. The active site of the 50S subunit is the peptidyl-transferase center (PTC) – the site of peptide bond formation. There are three tRNA binding sites on the 70S ribosome, the A-, P-, and E-sites. Peptide-bond formation occurs with a peptidyl-tRNA in the P-site and an aa-tRNA in the A-site. The E-site is the exit site, which the deacylated or uncharged tRNAs move through before exiting from the ribosome.

Protein synthesis encompasses four major phases: initiation, elongation, termination, and recycling (Schmeing

Table 1. Factors that interact with the ribosome under stress conditions

Factor	Stress/condition	Function
ArfA (YhdL)	mRNA damage	ArfA binds to ribosomes stalled on mRNAs lacking a stop codon and recruits RF2 to the A-site to hydrolyze the ester linkage of the peptidyl-tRNA
ArfB (YaeJ)	mRNA damage	ArfB binds to ribosomes stalled on mRNAs lacking a stop codon and catalyzes peptidyl-tRNA hydrolysis
BipA (TypA)	Antimicrobial peptides, cold shock, low pH, oxidative and detergent stress	Unknown, but BipA probably regulates translation of a specific subset of mRNAs under specific stress conditions
EF4 (LepA)	Potassium tellurite (oxidative stress), penicillin G, High Mg^{2+}	EF4 is proposed to bind to ribosomes stalled during a non-productive translocation and back-translocate the ribosome so translation can continue
EF-P	Ribosome stalling	EF-P binds to ribosomes stalled at di- and poly-prolyl motifs and stimulates peptide bond formation allowing translation to resume
EttA (YjjK)	ADP/ATP ratio	EttA senses the ADP/ATP ratio in the cell and under high ADP concentrations binds to the E-site of the ribosome to inhibit translation initiation
FusB/FusC	Antibiotic stress	FusB and FusC bind to EF-G trapped on the ribosome by the antibiotic fusidic acid and promote dissociation of EF-G, thereby enabling translation to continue and conferring resistance to fusidic acid
HPF (YrfH)	Stationary phase	The hibernation promotion factor binds to RMF-induced 90S disomes promoting formation of translationally inactive 100S particles. In some bacteria, the long-form HPFs stimulate 100S formation in the absence of RMF
Hsp15	Heat shock	Hsp15 binds to dissociated 50S subunits bearing peptidyl-tRNA in the A-site and stabilizes the peptidyl-tRNA in the P-site
MazF	Stationary phase	The MazF toxin cleaves mRNA and rRNA at ACA sequences removing the SD and anti-SD sequences, respectively, re-programming translation. The antitoxin MazE inactivates the toxin MazF
Obg	Stationary phase	Obg is involved in a late stage of ribosome biogenesis and acts as a negative regulator of SpoT activity
pY (YfiA, RaiA)	Stationary phase	pY binds and inactivates 70S ribosomes in stationary phase and under conditions of cold shock
RelA	Nutrient deprivation	The stringent factor RelA binds to ribosomes containing uncharged or deacylated tRNA at the A site and synthesizes the alarmone (p)ppGpp
RelE	Stationary phase	The RelE toxin cleaves mRNA at the A-site of the ribosome leading to 3′ truncated mRNAs lacking a stop codon. The antitoxin RelB inactivates RelE
RMF	Stationary phase	The ribosome-modulation factor (RMF) inactivates ribosomes by promoting formation of 90S disomes
RsfS (YbeB)	Stationary phase/Nutrient poor media	The ribosome-silencing factor S (RsfS/RsfA) binds to the L14 protein of the 50S subunit and impairs subunit joining
SmpB	mRNA damage	Small protein B (SmpB) binds tmRNA and is involved in the trans-translation system for rescue of ribosomes stalled on truncated mRNAs
SRA (S22)	Stationary phase	The stationary phase ribosome-associated protein, previously termed ribosomal protein S22, binds and inactivates ribosomes during stationary phase
TetM, TetO	Antibiotic stress	The RPPs TetM/TetO bind to ribosomes and dislodge tetracycline from the small subunit, thereby conferring resistance
YqjD	Stationary phase	An inner membrane protein that associates with 70S and 100S ribosomes and is proposed to localize ribosomes to the membrane during stationary phase

& Ramakrishnan, 2009). During initiation, the initiator fMet-tRNA is positioned with the start codon of the mRNA at the P-site of the ribosome. This process is facilitated by the initiation factors IF1, IF2, and IF3. The elongation phase involves the delivery of the aa-tRNA by elongation factor EF-Tu·GTP to the A-site of the ribosome. Correct interaction between the anticodon of the aa-tRNA and the A-site codon of the mRNA triggers hydrolysis of GTP to GDP by EF-Tu and subsequent dissociation of EF-Tu·GDP from the ribosome. The aa-tRNA accommodates into the PTC on the 50S subunit and undergoes peptide-bond formation with the

initiator-tRNA in the P-site (or peptidyl-tRNA in the subsequent round of elongation) resulting in a deacylated tRNA in the P-site and a peptidyl-tRNA in the A-site. Binding of elongation factor EF-G catalyzes the translocation reaction, which moves the mRNA and tRNAs through the ribosome, namely from the A- and P-sites into the P- and E-sites. Hydrolysis of GTP to GDP by EF-G leads to conformational changes in EF-G that promote the dissociation of EF-G·GDP from the ribosome. Translocation also moves a new codon into the A-site, dictating which aa-tRNA is delivered next. The appearance of a stop codon in the A-site signals the termination of translation. Stop codons are recognized by the release factors, RF1 or RF2, which catalyze release of the polypeptide chain by hydrolyzing the ester linkage to the P-tRNA. This post-termination ribosome is then dissociated into ribosomal subunits by the concerted action of the ribosome recycling factor (RRF) and EF-G, thus recycling the components for the next round of translation.

The ribosomal response to stress induced by nutrient deprivation

The stringent response links nutrient starvation, especially the shortage of amino acids, with all levels of gene expression, including changes in transcription, translation, and replication. This process is initiated by the stringent factor, an enzyme of the RelA/SpoT family, which synthesizes the hormone-like molecules ppGpp (guanosine 5′, 3′-(bis)diphosphate) and pppGpp (guanosine 5′-triphosphate, 3′-diphosphate) from ATP and GTP, collectively referred to as (p)ppGpp (Fig. 1a) (Somerville & Ahmed, 1979; Atkinson *et al.*, 2011). In a subset of Gram-negative α- and γ-*Proteobacteria*, (p)ppGpp is synthesized by RelA and/or SpoT but hydrolyzed only by SpoT, while the bifunctional enzyme, Rel/Spo, which both synthesizes and hydrolyzes (p)ppGpp, has a broader distribution being present in α/δ/ε-*Proteobacteria*, *Acidobacteria*, *Synergistetes*, *Aquificae*, *Bacteroidetes*, *Chlorobi*, *Spirochaetes*, *Actinobacteria*, *Cyanobacteria*, *Chloroflexi*, *Deinococcus/Thermus*, *Firmicutes*, *Tenericutes*, *Fusobacteria* (Atkinson *et al.*, 2011).

The pentaphosphate pppGpp, a less potent effector (Mechold *et al.*, 2013), normally emerges as the first product, which is then rapidly converted to ppGpp by a 5′ phosphohydrolase (Somerville & Ahmed, 1979). Accumulation of the two effector molecules, which are also called alarmones, is rapid, reaching millimolar concentrations in the cell within minutes. Both alarmones bind to the β-subunit of the *E. coli* polymerase (Chatterji *et al.*, 1998), which causes an immediate inhibition of transcription of components of the transcription and translation apparatus such as rRNAs, r-proteins, synthetases, tRNAs etc. (Dennis & Nomura, 1974), whereas metabolic

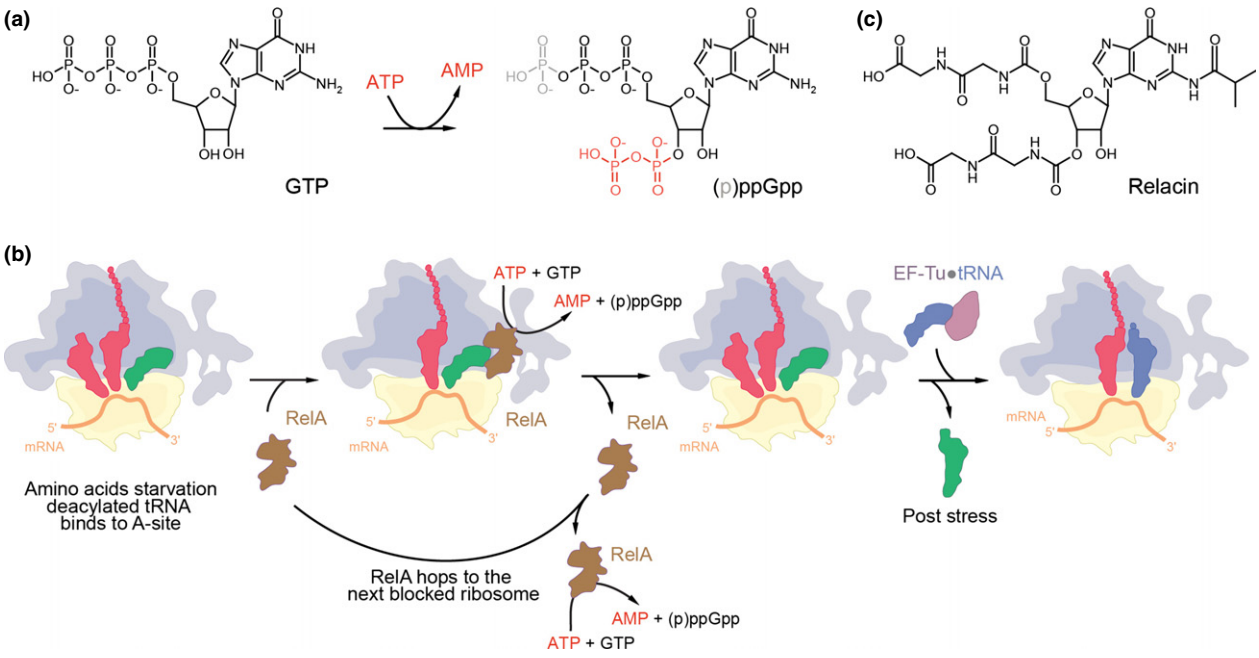

Fig. 1. Ribosome-dependent synthesis of ppGpp by RelA. (a) RelA catalyzes conversion of ATP and GTP (or GDP) to AMP and (p)ppGpp. (b) Scheme for RelA recognition of ribosomes stalled by deacylated tRNA and production of (p)ppGpp. (c) Chemical structure for RelA inhibitor Relacin (Wexselblatt *et al.*, 2012, 2013).

enzymes, particularly those involved in amino acid biosynthesis, are upregulated (Zhou & Jin, 1998). The process of (p)ppGpp formation is induced in response to elevated levels of uncharged (or deacetylated) tRNAs in the cell. These uncharged tRNAs bind non-enzymatically (i.e. without involvement of EF-Tu) to a vacant ribosomal A-site if the corresponding aminoacylated species is unavailable, thereby stalling translation (Fig. 1b). RelA recognizes the arrested ribosomes and catalyzes the synthesis of (p)ppGpp. The synthesis of (p)ppGpp by RelA is proposed to reduce its affinity for the ribosome, such that RelA is released and can then 'hop' to the next-blocked ribosome, thus repeating the cycle (Wendrich et al., 2002) (Fig. 1b). ppGpp has been recently shown to dramatically increase the turnover rate of its own ribosome-dependent synthesis by RelA, representing one of the rare examples of a positive allosteric regulation of an enzyme by its product (Shyp *et al.*, 2012). However, single molecule experiments in living cells indicate that RelA remains off the ribosome for extended periods of time after activation, suggesting that ppGpp synthesis by RelA also occurs in the absence of the ribosome, and therefore rebinding to the ribosome is not strictly required to trigger each (p)ppGpp synthesis event (English *et al.*, 2011). Upon amino acid availability, the deacetylated tRNAs are displaced by aa-tRNAs (Fig. 1b), which have higher binding affinities to the A-site than their uncharged counterparts (Wendrich *et al.*, 2002).

To date, there is relatively little structural information related to the stringent response machinery. The crystal structure of the hydrolase and synthetase domains from the bifunctional Rel/Spo from *Streptococcus dysgalactiae* has been solved (Hogg *et al.*, 2004). Based on this structure, inhibitors, such as Relacin have been designed (Wexselblatt *et al.*, 2012). Relacin is a 29-deoxyguanosine-based analogue of ppGpp, in which the original pyrophosphate moieties at 5′ and 3′ positions were replaced by glycyl-glycine dipeptides linked to the sugar ring by a carbamate bridge (Fig. 1c). Such analogues inhibit the synthetic activity *in vitro* of RelA and Rel/Spo proteins from both Gram-positive and Gram-negative bacteria (Wexselblatt *et al.*, 2012, 2013). Recently, a three-dimensional cryo-EM structure of the 70*S*-RelA complex revealed that the binding site of RelA on the ribosome overlaps with the canonical translation factor binding site, for example, of EF-Tu and EF-G (Agirrezabala *et al.*, 2013). The N-terminal domain of RelA was suggested to interact with the small subunit as well as with the acceptor arm of a tRNA in the ribosomal A-site. Interestingly, the A-tRNA was not observed bound in the classical state but adopts a distorted tRNA configuration similar to the A/T-tRNA conformation observed when a tRNA is initially delivered to ribosome by EF-Tu. In this conformation, the anticodon stem interacts with the decoding site of the small subunit, whereas the acceptor arm interacts with RelA (Fig. 1b). The distorted conformation of the A/T-tRNA results in contact between the elbow region of the tRNA and the L11-region of the ribosome (Agirrezabala *et al.*, 2013). Thus, the inhibition of (p)ppGpp synthesis observed in the absence of L11 (Wendrich *et al.*, 2002) or in the presence of antibiotics, such as thiostrepton that target the L11 region (Jenvert & Schiavone, 2005), may be due to hindrance of the tRNA to adopt the A/T conformation, rather than a direct effect on RelA. Unfortunately, the N-terminal synthetase domain was poorly ordered in the RelA-70S structure (Agirrezabala *et al.*, 2013), preventing any fitting of the crystal structure of this domain (Hogg *et al.*, 2004). Thus, exactly how interaction between RelA, the A/T-tRNA and the ribosome leads to stimulation of (p)ppGpp synthesis remains enigmatic.

The stringent control phenomenon, more or less denotes a situation of very high (p)ppGpp concentrations. However, (p)ppGpp signaling generally promotes survival under unfavorable conditions, and it is not surprising that (p)ppGpp signaling affects social behaviors, such as cell-cell communication, virulence, pathogen-host interaction, programmed cell death and persistence (Wagner, 2009). Persistence refers to a phenomenon in which individual cells of an isogenic antibiotic-sensitive population become multidrug tolerant. Recently, an attractive model of the molecular basis of persister cells was proposed: HipA of *E. coli*, a serine–threonine kinase, phosphorylates and thereby inactivates the glutamyl-tRNA synthethase GltX (Germain *et al.*, 2013). Consequently, binding of uncharged $tRNA^{Glu}$ to the ribosomal A-site trigger synthesis of (p)ppGpp. This is an interesting example where a natural stress response mechanism has evolved into a defense mechanism operating under antibiotic stress.

The ribosomal response to stress induced by peptide-mediated translational stalling

Not all amino acids are incorporated into the growing nascent polypeptide at the same rate: One notable exception is proline, which is unique among the 20 proteinogenic amino acids. Firstly, proline has an imino (rather than amino) group making it a poor A-site acceptor, as evidenced by the ribosome-catalyzed peptidyl transfer to $Pro\text{-}tRNA^{Pro}$ in the A-site being the slowest of all the tRNAs tested (Fig. 2a) (Pavlov *et al.*, 2009; Johansson *et al.*, 2011). Secondly, proline is also a poor donor when present in the P-site (Fig. 2a): $Pro\text{-}tRNA^{Pro}$ in the P-site displays exceptionally low reactivity with puromycin, an antibiotic that mimics the terminal adenosine A76 of the CCA-end of Tyr-tRNA (Mao, 1973; Muto & Ito, 2008;

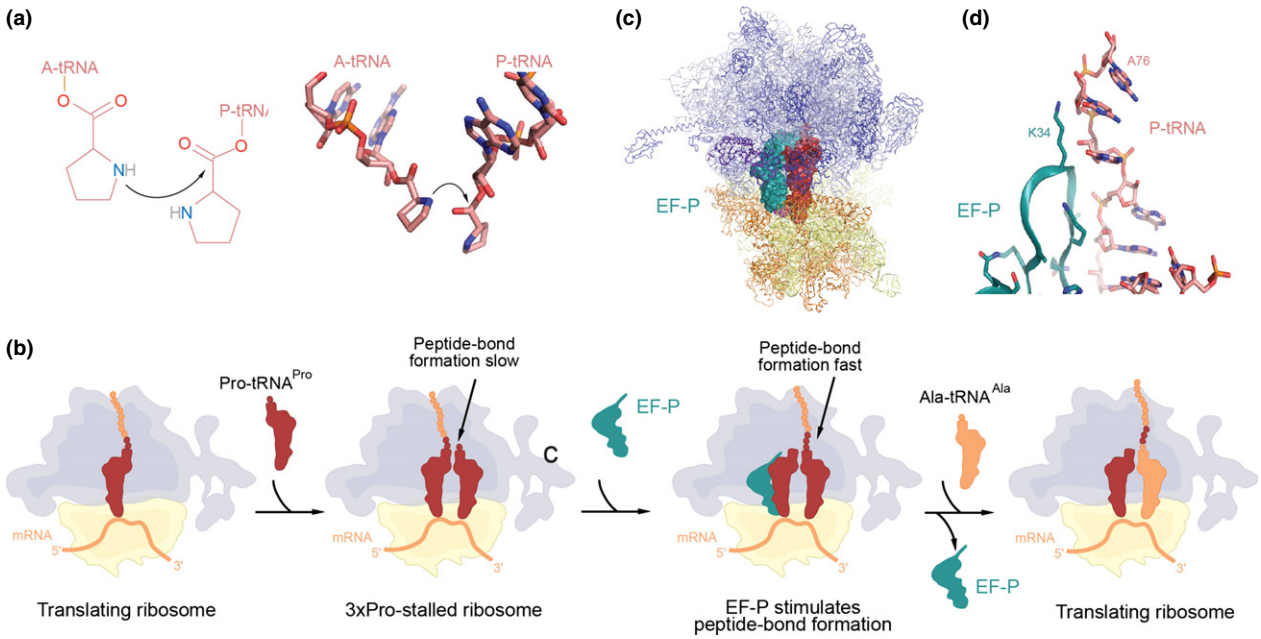

Fig. 2. Rescue of ribosomes stalled on polyproline stretches by EF-P. (a) Peptide-bond formation between Pro-tRNA in A- and P-site is slow because Pro acts as a poor donor and acceptor. (b) Ribosome stalling on polyproline stretches is alleviated by binding of EF-P, which stimulates peptide-bond formation so that translation can continue. (c) Binding site of EF-P on the 70S ribosome (Blaha *et al.*, 2009), with (d) enlargement indicating the position of the conserved lysine 34 in *Escherichia coli* EF-P relative to the CCA-end of the P-tRNA [based on (Blaha *et al.*, 2009)].

Wohlgemuth *et al.*, 2008; Doerfel *et al.*, 2013). In bacteria, ribosome pausing during translation elongation or termination results in transfer-messenger RNA (tmRNA)-mediated tagging (see The MazEF toxin–antitoxin system) (Hayes *et al.*, 2002; Tanner *et al.*, 2009; Woolstenhulme *et al.*, 2013). A single C-terminal proline residue is sufficient to induce tmRNA-mediated tagging at stop codons where it does not normally occur (Hayes *et al.*, 2002). Moreover, the efficiency of tagging can be influenced by the −2 position, with tandem Pro residues before the stop codon resulting in one of the largest impairments in termination efficiency (Hayes *et al.*, 2002). A subsequent genetic selection study *in vivo* identified three classes of sequences that induce translational stalling and subsequent tmRNA-tagging, namely, C-terminal Pro residues, SecM-like peptides, and a novel FxxYxIWPP(P)-stalling sequence (Tanner *et al.*, 2009). *In vitro*, it has been demonstrated that ribosomes stall when translating three or more consecutive proline residues (Doerfel *et al.*, 2013; Ude *et al.*, 2013; Woolstenhulme *et al.*, 2013). The translational stalling occurs when the peptidyl-Pro-Pro-tRNA is located in the P-site (Doerfel *et al.*, 2013; Woolstenhulme *et al.*, 2013) and results from slow peptide bond formation with the Pro-tRNA located in the A-site (Fig. 2b) (Doerfel *et al.*, 2013). Slow peptide-bond formation is also seen when Gly-tRNA is present in the A-site (Doerfel *et al.*, 2013). Consistently, while polyproline stretches produce the strongest translational stall, ribosome stalling is also observed with diprolyl motifs, namely, Pro-Pro-Gly (PPG), PPD, PPE, PPN, and PPW (Woolstenhulme *et al.*, 2013). Recently, a systematic analysis identified a distinct hierarchy of stalling triplets, ranging from strong stallers, such as PPP, DPP, and PPW to weak stallers, such as CPP, PPR, and PPH (Peil *et al.*, 2013). Moreover, tripeptide motifs (PPD, PPE and PPP) were shown recently to stall ribosomes in eukaryotic cells (Ingolia *et al.*, 2009; Gutierrez *et al.*, 2013), suggesting that stalling at polyproline stretches may be a general phenomenon, not limited to bacteria. The tripeptide motif PPP alone occurs *c.* 100 times in *E. coli* and the translation *in vitro* of many of these polyproline-containing proteins (so far tested: AmiB, CadC, FlhC, Flk, EF4, LigT, NlpD, RzoR, TonB, UvrB, and YafD) has been demonstrated to result in significant translational stalling (Doerfel *et al.*, 2013; Ude *et al.*, 2013; Woolstenhulme *et al.*, 2013), raising the question as to how these polyproline-containing proteins are translated *in vivo*.

Recently, it has been demonstrated in bacteria that a specialized translation elongation factor, EF-P, relieves translational stalling at polyproline-stretches *in vivo* (Ude *et al.*, 2013; Peil *et al.*, 2013) and *in vitro* (Doerfel *et al.*, 2013; Ude *et al.*, 2013). The slow rate of peptide-bond formation between a peptidyl-Pro-Pro-tRNA in the P-site

with the incoming Pro-tRNA in the A-site leads to ribosome stalling, which is recognized by EF-P (Fig. 2b). EF-P binds to the ribosome and stimulates the rate of peptide formation, relieving stalling and allowing translation to continue (Fig. 2b). Importantly, the ability of *E. coli* EF-P to relieve the translation arrest at polyproline clusters is dependent on lysinylation of EF-P by YjeA and YjeK (Doerfel *et al.*, 2013; Ude *et al.*, 2013). YjeK is a lysine 2,3-aminomutase that converts (S)-α-lysine into (R)-β-lysine (Behshad *et al.*, 2006; Peil *et al.*, 2012), whereas YjeA mediates transfer of (R)-β-lysine onto K34 of EF-P (Navarre *et al.*, 2010; Yanagisawa *et al.*, 2010; Peil *et al.*, 2012). YjeA is homologous to a lysine tRNA synthetase; however, it lacks the N-terminal codon recognition domain (Bailly & de Crecy-Lagard, 2010). Thus, YjeA acts like a tRNA synthetase to activate lysine (preferentially (R)-β-lysine) with ATP; however, it does not transfer the lysine to tRNA (Ambrogelly *et al.*, 2010; Yanagisawa *et al.*, 2010), but rather to the ε-amino group of K34 of EF-P (Navarre *et al.*, 2010; Yanagisawa *et al.*, 2010; Peil *et al.*, 2012). Unmodified EF-P is inactive and cannot alleviate translation arrest at polyproline clusters, whereas lysinylated EF-P (+ 128 Da) and hydroxylysinylated EF-P (+ 144 Da) are both equally active (Doerfel *et al.*, 2013; Ude *et al.*, 2013).

On the ribosome, EF-P spans both subunits and is positioned on the E-site side of the P-tRNA, with the site of lysinylation in domain I (K34) reaching towards the CCA-end of the P-tRNA (Fig. 2c and d) (Blaha *et al.*, 2009). A mechanistic understanding of exactly how the modified EF-P stimulates peptide bond formation is still lacking. Phylogenetic analyses show that EF-P has a ubiquitous distribution, being conserved in all bacteria and having homologs in archaea and eukaryotes (Bailly & de Crecy-Lagard, 2010). Recently, the yeast homolog of EF-P, eIF-5A, has been shown to function analogously to EF-P by relieving translation stalling at polyproline stretches (Gutierrez *et al.*, 2013). Genomic analyses reveal an abundance of polyproline stretches, not only in bacteria but also in archaea and eukaryotes. Therefore, stress conditions that require expression of large numbers of polyproline-containing proteins will require the presence of active EF-P or a/eIF-5A. The absence of *efp*, *yjeK*, or *yjeA* genes has been shown to cause defects in growth (Yanagisawa *et al.*, 2010; Iannino *et al.*, 2012; Zou *et al.*, 2012), fitness (de Crecy *et al.*, 2007), membrane integrity (Iannino *et al.*, 2012; Zou *et al.*, 2012), stress response (Ude *et al.*, 2013), sporulation (Ohashi *et al.*, 2003), motility (Kearns *et al.*, 2004; Inoue *et al.*, 2007; Zou *et al.*, 2012), antibiotics sensitivity, colonization, and virulence (Kaniga *et al.*, 1998; Peng *et al.*, 2001; Merrell *et al.*, 2002; Bearson *et al.*, 2006, 2011; Navarre *et al.*, 2010; Iannino *et al.*, 2012) in a variety of bacteria.

Ribosome-dependent toxin–antitoxin (TA) modules

TA systems are plasmid-borne or chromosomally encoded modules that can be found in all three domains of life. In general, TA systems comprise a minimum of two genes, one encoding a stable globular toxin protein and the second encoding a labile antitoxin protein that wraps around the toxin to inactivate it. Often, the toxin gene is located downstream to the antitoxin gene to ensure the presence of the antitoxin upon production of the respective toxin. Subsequent degradation of the antitoxin leads to the release of the toxin into the cell. TA systems function in bacterial programmed cell death, but also work as a control mechanism for microorganisms to cope with nutritional stress (Gerdes *et al.*, 1997, 2005) as well as in bacterial persistence (Maisonneuve *et al.*, 2013; Maisonneuve & Gerdes, 2014). Depending on its genetic organization, the mode of action and the kind of antitoxin, TAs can be divided into TA types I–V based on characteristics of the antitoxin (Schuster & Bertram, 2013). In line with the scope of this review, we focus on type II TA-systems that target the ribosome, such as RelBE and YefM/YoeB. We also included the well-studied MazEF system that cleaves mRNA in a ribosome-independent fashion because it also binds to the ribosome and cleaves the rRNA (Vesper *et al.*, 2011; Moll & Engelberg-Kulka, 2012). However, we note that a number of other type-II TA systems that target the translational machinery exist, although they are less well characterized: For example, as mentioned *E. coli* HipA phosphorylates and inactivates the glutamyl-tRNA synthethase GltX upon $tRNA^{Glu}$-binding (Germain *et al.*, 2013), the VapC toxin alters initiator tRNA molecules promoting translation initiation at elongator codons of otherwise silent genes (Winther & Gerdes, 2011), the *E. coli* RatA toxin interacts with the 50S subunit to prevent subunit joining (Zhang & Inouye, 2011) and the Doc toxin inhibits translation elongation (Liu *et al.*, 2008) via phosphorylation of EF-Tu (Castro-Roa *et al.*, 2013).

The MazEF toxin–antitoxin system

The type-II MazEF TA-systems are thought to most commonly target and cleave cellular mRNAs and are thus referred to as mRNA interferases (Inouye, 2006). Unlike the ribosome-dependent interferases that exclusively degrade mRNA associated with the ribosome (such as RelE and YoeB), the MazEF TA-system cleaves mRNAs at 3-, 5- or 7- nucleotide sequence motifs in the absence of the ribosome (Cook *et al.*, 2013). MazEF is one of the most intensively studied chromosomal TA-systems and was the first example that is regulated by ppGpp

(Aizenman *et al.*, 1996). *MazEF* loci are located downstream relative to the *relA* gene (Aizenman *et al.*, 1996), and are widely distributed among Gram-negative and Gram-positive bacteria (Pandey & Gerdes, 2005). The impact of MazEF on bacterial physiology remains controversial. While some studies suggest the role of MazEF in programmed cell death (Aizenman *et al.*, 1996; Amitai *et al.*, 2004; Hazan *et al.*, 2004), other studies suggest that the effects of the MazF toxin on bacteria are not bactericidal, but rather bacteriostatic, and can be reversed by expression of MazE antitoxin (Pedersen *et al.*, 2002; Christensen & Gerdes, 2003). The name *mazE* originates from the Hebrew language, *ma-ze*, meaning 'What is it?' (Metzger *et al.*, 1988). *MazE* encodes a labile antitoxin that antagonizes the stabile toxin MazF by forming a linear heterohexamer, such that the C-terminal extensions of each MazE wrap around a respective MazF dimer (Kamada *et al.*, 2003; Simanshu *et al.*, 2013) (Fig. 3a). Comparison with the structure of MazF in complex with mRNA (Fig. 3b and c) reveals that the C-terminal MazE extensions encroach the mRNA binding site on the MazF dimer, thus explaining how the MazE antitoxin inactivates the MazF toxin (Simanshu *et al.*, 2013). The *E. coli* MazF mRNA interferase cleaves free single-stranded mRNAs both *in vitro* and *in vivo*, preferentially but not exclusively, at ACA sequences (5′ to the C), leaving a 2′ 3′-cyclic phosphate group at one side and a free 5′-hydroxyl group at the other side (Zhang *et al.*, 2003, 2004). However, mRNAs bound to translating ribosomes are cleaved more efficiently, probably due to removal of secondary structure by the ribosome (Christensen *et al.*, 2003; Zhang *et al.*, 2003).

MazE and *mazF* are co-expressed, and transcriptionally, feedback inhibit their own synthesis (Marianovsky *et al.*, 2001). Numerous stress conditions, such as antibiotic treatment, heat, phage infection, oxidative stress, as well as an increase in ppGpp levels upon severe amino acid starvation, trigger activation of the MazEF TA-system (Hazan *et al.*, 2004). The ATP-dependent ClpAP serine protease, or the Lon protease under stress conditions, cleave MazE, thus freeing the stable MazF toxin to exert its function (Aizenman *et al.*, 1996; Christensen *et al.*, 2003). MazF cleavage of mRNAs in turn leads to the inhibition of the synthesis of *c.* 90% of all cellular proteins (Amitai *et al.*, 2009). However, the synthesis of some proteins (mostly < 20 kDa in size) tends to be increased (Amitai *et al.*, 2009). Among these one can find translation elongation factor EF-P, which alleviates ribosome stalling during translation of nascent polypeptide chains containing polyproline stretches (see The ribosomal response to stress induced by peptide-mediated translational stalling). In this respect, it is notable that MazF

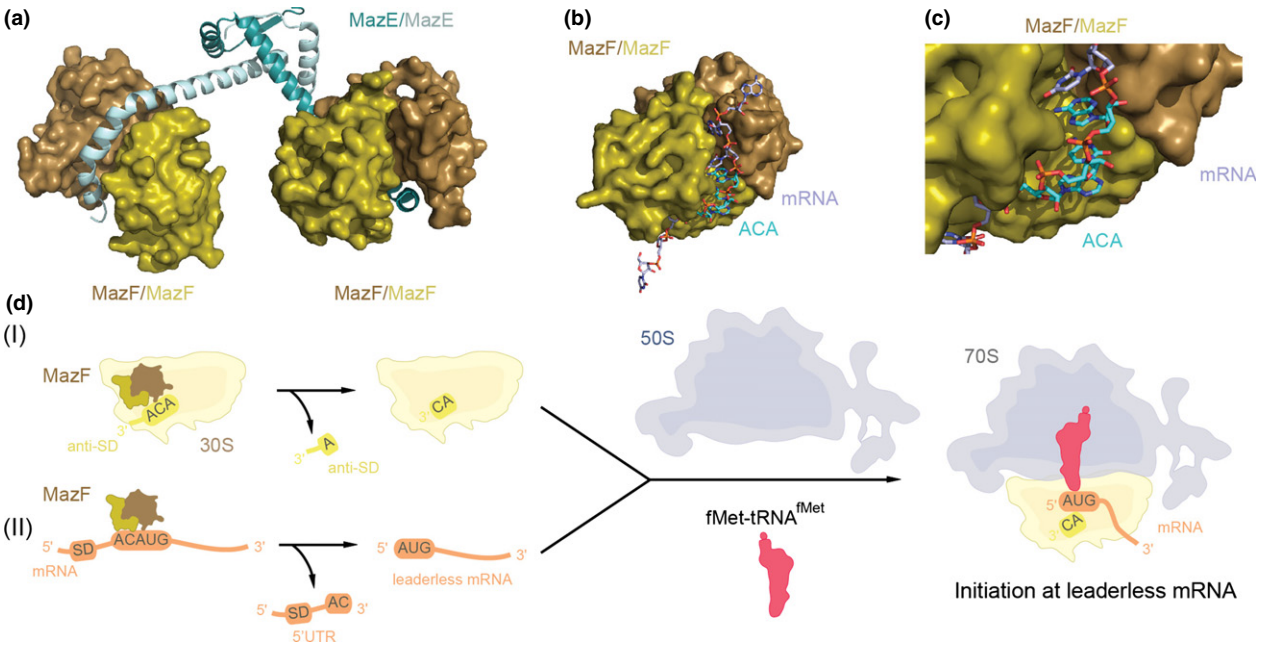

Fig. 3. MazF-mediated translation reprogramming. (a–c) Structure of the *Escherichia coli* toxin MazF dimer in complex with (a) the antitoxin MazE or (b) mRNA, with (c) enlargement of ACA (cyan) of mRNA in active site (Simanshu *et al.*, 2013). (d) Scheme for MazF cleavage of ACA motif in (I) the 16S rRNA of the 30S subunit, thus removing the anti-SD, and (II) mRNA, adjacent to the AUG start codon, thus generating a leaderless mRNA lacking SD sequence, which is specifically translated by the MazF cleaved ribosomes.

also targets the tmRNA [by the less common cleavage of the stop codon UAA as well as other sense codons, (Christensen & Gerdes, 2003)], a general rescue system on stalled ribosomes (see The ribosomal response to stress induced by mRNA truncation). Taken together, one might speculate that decreased tmRNA levels and increased EF-P levels further support translation of specific proteins.

In the study of Vesper *et al.* (2011), *E. coli* MazF was found to preferentially cleave at ACA sequences located at, or slightly upstream of, AUG start codons, resulting in leaderless or short-leadered mRNAs, respectively (Fig. 3d) (Moll & Engelberg-Kulka, 2012). These mRNAs are then specifically recognized by a subclass of so-called 'stress-ribosomes' that are also the product of the MazF endoribonuclease activity: Cleavage by MazF within the ribosomal 16S rRNA occurs at a distinct ACA motif, which leads to loss of a 43-nucleotide fragment at the 3′ end (Fig. 3d). This fragment encompasses helix 45 as well as the anti-Shine–Dalgarno sequence (anti-SD). As the anti-SD sequence is required for initiation of many canonical mRNAs via interaction with the corresponding SD sequence of the mRNA, the newly formed stress-ribosomes can selectively translate the leaderless mRNAs (Fig. 3d). Thus, the reported ribosome heterogeneity leads to a model for an elegant mechanism by which the cell can modulate protein production in favor of a specific subset of mRNAs (Moll & Engelberg-Kulka, 2012).

Interestingly, *Mycobacterium tuberculosis* has nine MazEF TA-systems that exhibit different sequence specificities for mRNA cleavage; recently, one of these, MazF-mt6, has been shown to also inactivate translation by cleavage of the 23S rRNA within the PTC of the 50S subunit (Schifano *et al.*, 2013).

The RelBE toxin–antitoxin system

In contrast to the ribosome-independent mRNA interferase activity of MazF, the mRNA cleavage activity of RelE is strongly dependent on ribosome binding (Pedersen *et al.*, 2003; Neubauer *et al.*, 2009). The chromosomal *relBE* locus (Bech *et al.*, 1985) encodes the toxin RelE and the corresponding antitoxin RelB. The crystal structure of the intact *E. coli* $RelB_2E_2$ TA complex reveals that the RelB antitoxin wraps around the RelE toxin (Fig. 4a) (Boggild *et al.*, 2012), which on one hand blocks access of mRNA to the active site of the RelE toxin (Fig. 4b) (Neubauer *et al.*, 2009), but also prevents entry into the ribosomal A-site (Fig. 4c). In general, RelB is targeted by the Lon protease, which degrades the antitoxin, leading to activation and ribosome binding of the previously inactive RelE toxin (Christensen *et al.*, 2001; Galvani *et al.*, 2001; Li *et al.*, 2009). RelE cleavage efficiency is influenced by the mRNA sequence, displaying a preference for UAG and UGA stop codons as well as UCG and CAG sense codons (Pedersen *et al.*, 2003).

Crystal structures of RelE in complex with the 70S ribosome reveal that RelE binds within the decoding site on the 30S subunit (Fig. 4c) (Neubauer *et al.*, 2009). To obtain mechanistic insights into RelE action, a precleavage state was obtained by using an inactive RelE-R45A/R81A mutant and an mRNA bearing 2′-O-methylated UAG codon in the A-site (Fig. 4d). This was then compared with postcleavage state where wildtype RelE was used in conjunction with mRNA containing an unmodified UAG codon (Fig. 4e). The precleavage state shows that RelE binding alters the path of the mRNA through the A-site (Fig. 4d), compared with when tRNA is bound (Fig. 4f). In the precleavage state, the bases in positions 2 (A20) and 3 (G21) of the A-site codon are splayed apart and stack upon a conserved tyrosine (Y87) in RelE and C1054 of the 16S rRNA, respectively (Fig. 4e). In the postcleavage state, A20 maintains stacking interaction with Y87 and the 3′ of the mRNA following the second position has been removed (Fig. 4e), consistent with biochemical data demonstrating that RelE cleaves between position 2 and 3 of the A-site codon of the mRNA (Pedersen *et al.*, 2003; Neubauer *et al.*, 2009). Moreover, the data support a model in which RelE-mediated cleavage of mRNA occurs after position 2 of the A-site codon by a 2′-OH-induced hydrolytic mechanism, leading to formation of a 2′-3′ cyclic phosphate at the newly formed 3′ end (Fig. 4e) (Neubauer *et al.*, 2009). Mutagenesis studies indicate that mutations R61A or R81A significantly lower the cleavage activity of RelE, suggesting a role for these residues as a general base to abstract a proton from 2′ OH and thus activating it for nucleophilic attack (Fig. 4d and e). In this model, Tyr87 and C1054 are also important for orienting the substrate correctly for the nucleophilic attack (Fig. 4d).

As a consequence of RelE-mediated cleavage, translation cannot continue due to the presence of a truncated mRNA in the A-site (Fig. 4g). Because of this, translation rates during amino-acid starvation decrease. This, in turn, might offer several advantages to the organism: On the one hand, nutrient and energy consumption rates can be adjusted rapidly in accordance with supply limitations, whereas on the other hand, diminished translation and hence higher tRNA charging would result in lower levels of translational errors (O'Farrell, 1978; Nystom, 1994; Gerdes *et al.*, 2005). Beside Lon-dependent toxin activation and the link to the stringent response, RelE is counteracted by tmRNA (Christensen & Gerdes, 2003; Christensen *et al.*, 2003) and underlies an auto-regulated circuit based on transcriptional repression of the *relBE*

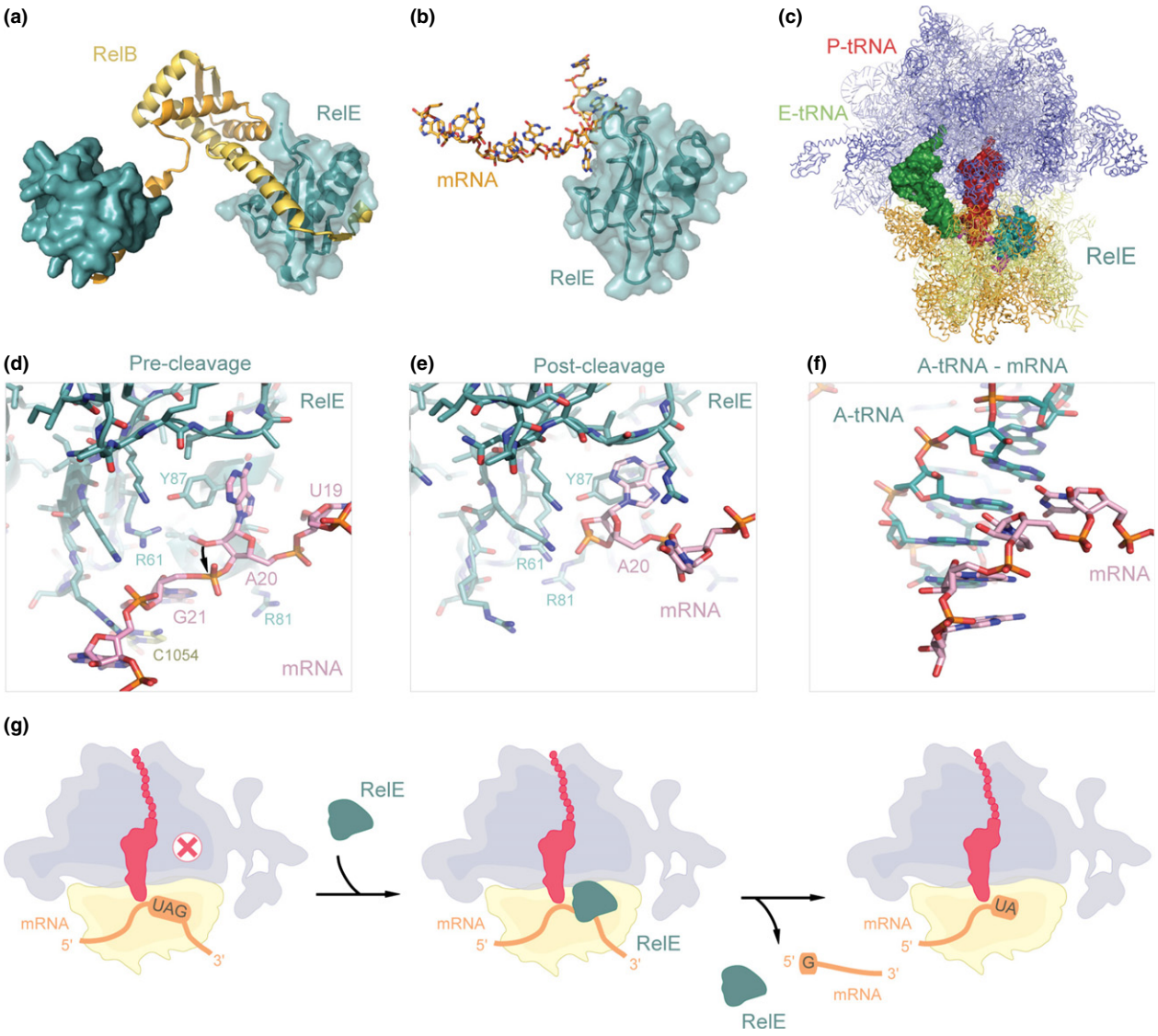

Fig. 4. Ribosome-dependent cleavage of the A-site codon by RelE. (a and b) Structure of the *Escherichia coli* toxin RelE in complex with (a) the antitoxin RelB (Boggild *et al.*, 2012) or (b) mRNA (Neubauer *et al.*, 2009). (c) Binding site of RelE on the 70S ribosome (Neubauer *et al.*, 2009). (d) pre- and (e) post-cleavage states of mRNA by RelE on the 70S ribosome, compared with (f) mRNA conformation in the presence of A-tRNA (Neubauer *et al.*, 2009). (g) Scheme for RelE mediated mRNA cleavage on the ribosome.

promoter by RelBE and RelE in a ratio-dependent manner (Overgaard *et al.*, 2008, 2009; Boggild *et al.*, 2012; Cataudella *et al.*, 2012).

Other ribosome-dependent TA systems in *E. coli*

In *E. coli* K-12, RelBE is not the only ribosome-dependent type-II TA-system. Four additional TA-systems have been reported, namely, YefM/YoeB, DinJ/YafQ, YafNO, and YgjNM (Gerdes, 2000; Gerdes *et al.*, 2005; Zhang *et al.*, 2009; Christensen-Dalsgaard *et al.*, 2010). All of them belong to the RelE superfamily and contribute to the translational stress response: *ygjNM* and *yafNO* are induced by amino acid starvation and inhibition of translation, although to differing degrees (Christensen-Dalsgaard *et al.*, 2010). In addition, the *yafNO* locus is also induced by DNA damage, whereas *ygjNM* is not (Christensen-Dalsgaard *et al.*, 2010). After induction, the YafO toxin exerts its function by interaction with the 50S subunit of the ribosome (Prysak *et al.*, 2009), which contrasts with RelE, but is similar to the YafQ toxin component of the DinJ/YafQ TA-system. Upon ribosome binding, YafQ reportedly cleaves the mRNA 5′ of the

third nucleotide of AAA codons followed by G or A, which in turn leads to a rapid decay of mRNA presumably via the mRNA degradosome (Prysak *et al.*, 2009; Armalyte *et al.*, 2012). However, the cleavage site is not stringent (Armalyte *et al.*, 2012) and thus it is possible that the position of the mRNA within the ribosome, rather than the sequence, directs the cleavage (Armalyte *et al.*, 2012). The DinJ/YafQ TA-system is transcriptionally activated by LexA after DNA damage (Prysak *et al.*, 2009; Armalyte *et al.*, 2012) and is also involved in multidrug tolerance in *E. coli* biofilms (Harrison *et al.*, 2009). Analogously, the first described example of a YefM/YoeB-like TA-system, the Axe/Txe TA-system allows development of multidrug-resistant *Enterococcus faecium* (Grady & Hayes, 2003). Here, Axe/Txe act like a functional segregational stability module on a plasmid pRUM, conferring resistance to chloramphenicol, erythromycin, streptomycin, and streptothricin (Grady & Hayes, 2003). YefM/YoeB-like TA-systems are widely distributed in bacteria and archaea among plasmids and genomes, whereas the *E. coli* TA-system is a chromosomal module. Similar to RelBE, the inactive form of the YefM/YoeB complex is presumably a heterotrimer. The YefM antitoxin is degraded presumably by the Lon protease under as-yet-unspecified stress conditions. Recently, the crystal structure of YoeB bound to 70S ribosome was solved and revealed that unlike RelE, YoeB binds as a dimer to the ribosome (Feng *et al.*, 2013). However, only one YoeB monomer is directly involved in cleavage, where the conserved Glu46 and His86 residues are employed for general acid-base catalysis, similar to other RNases. Nevertheless, the outcome is the same, namely, a direct nucleophilic attack of the 2′ OH of position 2 of the mRNA (A20) onto the phosphate of the nucleotide following the second A-site codon as observed in the RelE structure (Fig. 4d). Activation of such TA-loci, including mRNases MazF and RelE, by amino acid starvation, elevated levels of (p)ppGpp or other stress factors inhibits global protein synthesis which leads to induction of dormancy and persistence – phenomenona that play critical roles for cell survival, especially of pathogenic bacteria (Maisonneuve *et al.*, 2011; Maisonneuve & Gerdes, 2014).

The ribosomal response to the stress induced by mRNA truncation

Under normal circumstances, the translation process is terminated when a ribosome encounters a stop codon. At this point, specialized termination release factors bind to the ribosome and catalyze the release of the fully translated polypeptide chain (Schmeing & Ramakrishnan, 2009; Giudice & Gillet, 2013). However, the ribosome can also be stopped erroneously, with the most common reason being a ribosome stalling upon reaching the 3′ end of a non-stop mRNA, generally due to a preceding truncation of the mRNA (Janssen & Hayes, 2012). Such non-stop situations lead to non-productive translation complexes, comprising the trapped ribosome as well as the peptidyl-tRNA and mRNA. These complexes further accumulate into polysomes and can no longer be recruited for new rounds of translation (Janssen & Hayes, 2012). Although programmed ribosomal stalling is advantageous and used for regulation of gene expression (Kobayashi *et al.*, 2008; Ito *et al.*, 2010; Wilson & Beckmann, 2011), in most other situations, ribosome stalling has a deleterious effect on bacterial fitness (Doma & Parker, 2007; Janssen & Hayes, 2012). Interestingly, non-stop events occur frequently *in vivo* (Ito *et al.*, 2011), indicating that bacterial cells are well equipped with quality-control systems to maintain both the fidelity and speed of translation (Doma & Parker, 2007; Giudice & Gillet, 2013).

Trans-translation by tmRNA and SmpB

The first characterized response of bacteria to quality control of protein synthesis is referred to as trans-translation (Fig. 5a) (Moore & Sauer, 2007; Keiler, 2008). Here, bacteria deploy a complex composed of two molecules, namely the transfer-messenger RNA (tmRNA) and the small basic protein B (SmpB). The tmRNA (10Sa RNA), together with the catalytic subunit of RNase P (10Sb), were discovered in 1979 during a search for stable RNA molecules in *E. coli* (Ray & Apirion, 1979; Jain *et al.*, 1982). Ten years later, the corresponding gene for tmRNA was identified as *ssrA* (small stable RNA). *SsrA* defective cells show a pleiotropic phenotype with deficiencies in growth and carbon starvation; however, its function remained enigmatic (Oh & Apirion, 1991). Finally, in the mid-90′s, the tRNA-like nature of tmRNA was recognized (Komine *et al.*, 1994) and was shown to facilitate the tagging of truncated proteins with a carboxy-terminal degradation sequence (Tu *et al.*, 1995; Keiler *et al.*, 1996). The tmRNA is a hybrid tRNA-mRNA molecule comprising an alanine-charged tRNA-like domain (TLD) and a short internal mRNA-like domain (MLD) (Fig. 5b). The tmRNA-mediated ribosome rescue essentially depends on SmpB, which is encoded adjacent to *ssrA* in *E. coli* (Karzai *et al.*, 1999; Moore & Sauer, 2005). SmpB is *c.* 20 kDa and it's only known cellular role is related to trans-translation: *in vivo* SmpB binds to tmRNA with a high affinity and prolongs its half-life by *c.* threefold (Hallier *et al.*, 2004). Moreover, SmpB is needed for the stable association of tmRNA with arrested ribosomes (Hallier *et al.*, 2006), facilitating tmRNA charging by alanyl-tRNA

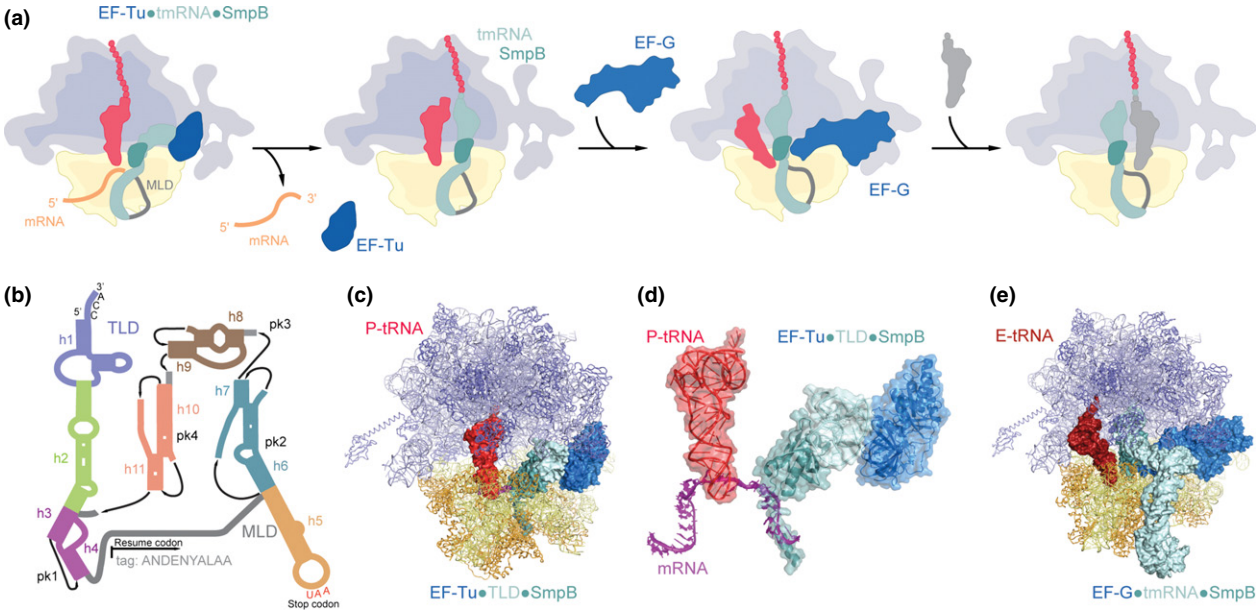

Fig. 5. Trans-translation of truncated mRNAs by tmRNA and SmpB. (a) Scheme for tmRNA-mediated rescue of ribosomes stalled on truncated mRNAs. (b) Secondary structure for tmRNA, with tRNA-like domain (TLD) and mRNA-like domain (MLD) highlighted. (c) Structure of EF-Tu delivery of tmRNA-TLD-SmpB complex to the A-site of the 70S ribosome (Neubauer *et al.*, 2012). (d) C-terminal domain (CTD) of SmpB overlaps position of A-site codon and 3′ extension of mRNA within the mRNA channel (Neubauer *et al.*, 2012). (e) Structure of complete tmRNA (cyan)-SmpB (teal) complex following translocation into the P-site by EF-G (blue) (Ramrath *et al.*, 2012).

synthetase (Barends *et al.*, 2001) and was shown to co-migrate with the tRNA module of tmRNA during translation of the mRNA module (Shpanchenko *et al.*, 2005).

Structures of SmpB reveal an oligonucleotide-binding (OB) fold built of six antiparallel β-strands forming a closed β-barrel that exposes two conserved RNA-binding sites (Dong *et al.*, 2002; Neubauer *et al.*, 2012). Trans-translation starts with the recognition of a ribosome containing an mRNA lacking or bearing an incomplete A-site codon (Moore & Sauer, 2007; Keiler, 2008). This is accomplished by a quarternary complex composed of alanine-charged tmRNA, SmpB, elongation factor EF-Tu and GTP. Recently, the crystal structure of a ribosome in complex with the tmRNA TLD, SmpB, and EF-Tu was solved (Neubauer *et al.*, 2012) (Fig. 5c). Together, the TLD and SmpB resemble the canonical L-shape of a tRNA molecule. In this complex, SmpB structurally mimics the anticodon stem of the A-tRNA and is located within the decoding site where codon-anticodon interactions take place (Gutmann *et al.*, 2003; Bessho *et al.*, 2007; Kurita *et al.*, 2007). Interestingly, the essential carboxy-terminal tail of SmpB, which is unstructured in solution (Dong *et al.*, 2002), becomes completely ordered upon ribosome-binding. The tail first extends from the SmpB core towards the decoding center and then continues along the pathway that is occupied by the 3′ end of the mRNA during canonical translation (Fig. 5d). As this interaction between the tail of SmpB and the stalled ribosome can only occur when the mRNA is truncated, this ensures that an overlap between canonical translation and trans-translation is prohibited (Neubauer *et al.*, 2012).

During canonical translation, the correct interaction between anticodon of the tRNA and codon of the mRNA induces domain closure within the 30S subunit. This in turn triggers GTP hydrolysis, release of EF-Tu·GDP from the ribosome, and subsequent accommodation of the aa-tRNA into the PTC on the 50S subunit. During trans-translation, SmpB interaction with the 30S subunit appears to stimulate domain closure with a stacking interaction between His136 of SmpB and G530 within the 16S rRNA playing a critical role (Neubauer *et al.*, 2012; Miller & Buskirk, 2014). Surprisingly, accommodation of the alanyl-tRNA-like domain of tmRNA into the PTC on the 50S subunit can even occur in the absence of GTP hydrolysis and release of EF-Tu (Miller & Buskirk, 2014). Peptide bond formation then transfers the truncated nascent polypeptide chain to the alanyl-TLD of the tmRNA (Fig. 5a). Cryo-EM structures have revealed how the tmRNA-SmpB complex accommodates into the A-site and is translocated by EF-G into the P-site (Fu *et al.*, 2010; Weis *et al.*, 2010; Ramrath *et al.*, 2012). In the translocated state, SmpB is located at the P-site on the

30S subunit, whereas the TLD is present at the P-site of the 50S subunit and the helical pseudoknot (pk2-4) structure wraps around the head of the small subunit (Fig. 5e). In this translocated state, the MLD of the tmRNA is now positioned such that the resume codon located in the A-site and directs delivery of the next aa-tRNA (Fig. 5a). The entire MLD of tmRNA can now be translated in the canonical fashion, which appends the encoded degradation tag onto the polypeptide chain. As the MLD of tmRNA also encodes a canonical stop codon, termination and recycling can occur and the ribosomes are then available for the next round of translation.

The alternative rescue factor ArfA (YhdL)

Until recently, trans-translation by tmRNA and SmpB was the only system reported for rescuing ribosomes stalled on truncated mRNAs in bacteria. Both tmRNA and SmpB are highly conserved among bacteria; however, deletions do not necessarily lead to a lethal phenotype, for example, *E. coli* Δ*ssrA* mutants are viable (Retallack & Friedman, 1995; Abe *et al.*, 2008; Liu *et al.*, 2010b). This suggested the existence of additional back-up mechanisms and indeed two alternative rescue factors ArfA (YhdL) and ArfB (YaeJ) were recently discovered (reviewed by Giudice & Gillet, 2013).

ArfA was identified in a screen where *ssrA* is crucial for survival of *E. coli* (Chadani *et al.*, 2010). The *arfA* gene encodes a small 72aa protein, which surprisingly is often not translated from the full-length mRNA, but rather from a mRNA fragment yielding a shortened ArfA protein of 55aa (Garza-Sanchez *et al.*, 2011). This fragment is generated by RNase III cleavage of a hairpin structure within the *arfA* mRNA, resulting in a transcript lacking a stop codon (Garza-Sanchez *et al.*, 2011). The functional C-terminally shortened ArfA protein is only produced in the absence of tmRNA-mediated trans-translation, when it is released from the ribosome and participates in the rescue of stalled ribosomes (Chadani *et al.*, 2011b; Garza-Sanchez *et al.*, 2011; Schaub *et al.*, 2012). Indeed, ArfA lacks the tripeptide sequence Gly-Gly-Gln (GGQ), which is essential for peptidyl-tRNA hydrolysis at the P site mediated by class I release factors RF1 or RF2 during canonical termination at stop codons (Chadani *et al.*, 2010). Therefore, ArfA-mediated rescue of ribosomes stalled on truncated mRNAs requires RF2 to be recruited to the ribosome to release the polypeptide chain from the P-tRNA (Chadani *et al.*, 2012). Consistently, mutation of the GGQ motif of RF2 involved in peptidyl-tRNA hydrolysis, but not the SPF (Ser-Pro-Phe) motif involved in decoding affects the ArfA-mediated rescue (Chadani *et al.*, 2012). ArfA has been proposed to bind in the A-site, analogously to SmpB, to recruit RF2 (Fig. 6a); however, at present there is no evidence that ArfA actually binds in the A-site, nor interacts directly with RF2. Similarly, it is unclear how the presence of deacylated and aa-tRNAs can stimulate ArfA-mediated recruitment of RF2 to the non-stop ribosomes. Pech & Nierhaus suggested that ArfA might bind to the E-Site, weakening codon–anticodon interactions to render the A-site prone to errors and hence RF2 could be recruited to the A site without a stop codon to relieve the arrested ribosome (Pech & Nierhaus, 2012).

The alternative rescue factor ArfB (YaeJ)

An alternative way to rescue stalled ribosomes is mediated by ArfB (Chadani *et al.*, 2011a; Handa *et al.*, 2011). In contrast to ArfA, which is restricted to *β*- and *γ*-*Proteobacteria*, ArfB homologs are also present in eukaryotes, for example, ICT1 acting on mitochondrial ribosomes (Handa *et al.*, 2011; Schaub *et al.*, 2012). With respect to sequence and structure, ArfB resembles the catalytic domain of class I release factors. This also includes the tripeptide motif GGQ, which is crucial for ArfB-mediated peptidyl-tRNA hydrolysis (Chadani *et al.*, 2011a). ArfB binds to ribosomes stalled on either non-stop mRNAs or mRNAs with rare codon clusters, and subsequently hydrolyzes the peptidyl-tRNA *in vitro* (Handa *et al.*, 2011). Recently, Gagnon *et al.* (2012) solved the crystal structure of ArfB bound to the ribosome, providing a structural basis for ArfB action (Fig. 6b): Similar to SmpB (Neubauer *et al.*, 2012), the carboxy-terminal domain of ArfB also acts as a sensor to distinguish between actively translating and arrested ribosomes based on the occupancy of the mRNA entry channel (Fig. 6c). Subsequently, the GGQ motif containing N-terminal catalytic domain of ArfB can be oriented optimally at the PTC to catalyze peptidyl-tRNA hydrolysis (Fig. 6d) (Savelsbergh *et al.*, 2009; Gagnon *et al.*, 2012), and then the ribosomes can be recycled by RRF and EF-G (Schmeing & Ramakrishnan, 2009). Although these findings provide mechanistic details into ArfB action on the ribosome, the physiological role of ArfB remains obscure (Chadani *et al.*, 2011a; Handa *et al.*, 2011; Giudice & Gillet, 2013): In contrast to *arfA*, an *E. coli* double deletion of *ssrA* and *arfB* is viable and the synthetic lethal mutant Δ*ssrA*/Δ*arfA* can be rescued by ArfB overexpression. Thus, one might speculate that ArfB is of importance only under special environmental conditions. In this context, Jiang *et al.* (2009) and Giudice & Gillet (2013) have suggested that ArfB might rescue heat shock aborted ribosomes in a concerted action with heat shock protein 15 (see Hsp15 and the ribosomal heat shock response).

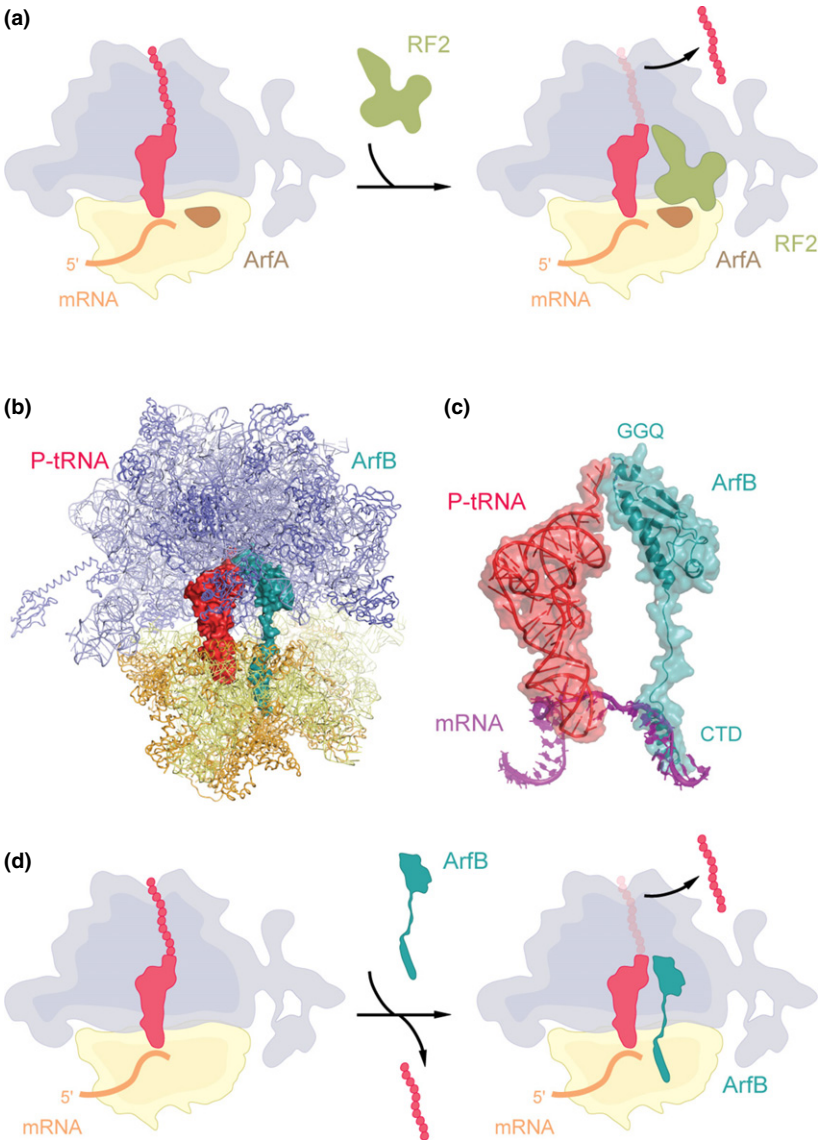

Fig. 6. Rescue of truncated mRNAs by ArfA and ArfB. (a) Schematic for ArfA-mediated recruitment of RF2 to the 70S ribosome. (b) Structure of ArfB on the 70S ribosome (Gagnon *et al.*, 2012). (c) The C-terminal domain (CTD) of ArfB overlaps position of A-site codon and 3′ extension of mRNA within the mRNA channel (Gagnon *et al.*, 2012). (d) Schematic for ArfB mediated ribosome rescue.

The ribosomal response to stationary phase

Bacterial growth is characterized by different growth phases. When nutrients become limiting and/or inhibitory products are accumulating, then bacteria shift from exponential growth into a stationary phase. In the stationary phase, morphological and physiological changes occur. These include the transformation of rod-shaped cells into small spherical cells with condensed nucleoids, which are resistant to many different forms of stress (Serra *et al.*, 2013). Such transformations are associated with changes in the expression of > 200 genes and proteins (Schellhorn *et al.*, 1998; Gutierrez-Rios *et al.*, 2003). Several of these proteins correspond to ribosome-binding proteins, including protein Y (pY, YfiA or earlier termed RaiA, the ribosome-associated inhibitor A), the ribosome modulation factor (RMF), the hibernation-promoting factor (HPF, YhbH), the energy-dependent translational throttle A (EttA, YjjK) as well as the stationary-phase-induced ribosome-associated protein (SRA) (Izutsu *et al.*, 2001b), RsfS (ribosome silencing factor S, YbeB) and the membrane-integrated ribosome-binding protein YjqD.

Protein Y inactivates the 70S ribosome

The protein factor Y was first identified as a protein spot (spot Y) on two-dimensional gels used to search for protein factors that interact with the 70S ribosome (Agafonov *et al.*, 1999). Protein Y (pY) was shown to be

encoded by the *E. coli yfiA* gene, bind to 30S subunits and stabilize 70S ribosomes against dissociation under conditions of low magnesium (Agafonov *et al.*, 1999; Vila-Sanjurjo *et al.*, 2004; Sharma *et al.*, 2010). *In vivo*, pY is expressed and binds to 70S ribosomes during stationary phase and under conditions of cold shock, but is rapidly released as soon as normal growth conditions are restored (Maki *et al.*, 2000; Agafonov *et al.*, 2001). Biochemically, pY inhibits translation by competing with binding of tRNAs to the A- and P-site of the 70S ribosome (Agafonov *et al.*, 2001; Vila-Sanjurjo *et al.*, 2004; Sharma *et al.*, 2010). Consistently, crystal structures of pY bound to the 70S ribosome reveal that pY binds to the 30S subunit of a 70S ribosome in a position overlapping the tRNAs and mRNA in the A- and P-site (Vila-Sanjurjo *et al.*, 2004; Polikanov *et al.*, 2012) (Fig. 7a and b). Moreover, an analogous binding site on the 70S ribosome was observed in cryo-EM studies for the chloroplast homologue of pY, originally termed plastid-specific ribosome protein 1 (PSRP1) (Sharma *et al.*, 2007, 2010). PSRP1 and *E. coli* pY also compete with the binding of initiation factor IF3 to the ribosome (Vila-Sanjurjo *et al.*, 2004; Sharma *et al.*, 2010), indicating that pY can block translation initiation under stress conditions. When growth conditions are restored, pY can be removed from the ribosome by EF-G and RRF, enabling the ribosomes to be returned to the active translation pool (Sharma *et al.*, 2010).

RMF and HPF cooperate to produce inactive 100S ribosome dimers

RMF is a small basic protein that is only present in γ-*Proteobacteria*, such as *E. coli* (Ueta *et al.*, 2008). RMF is detected in stationary phase *E. coli* cells and requires ppGpp for expression (Izutsu *et al.*, 2001a). In *E. coli*, RMF stimulates the dimerization of 70*S* ribosomes to form 90*S* particles (Wada *et al.*, 1995), which are converted to translationally inactive 100S particles by the additional binding of HPF (Ueta *et al.*, 2005, 2008) (Fig. 7c). Upon transfer from starvation conditions into fresh medium, RMF and HPF are released and the 100S

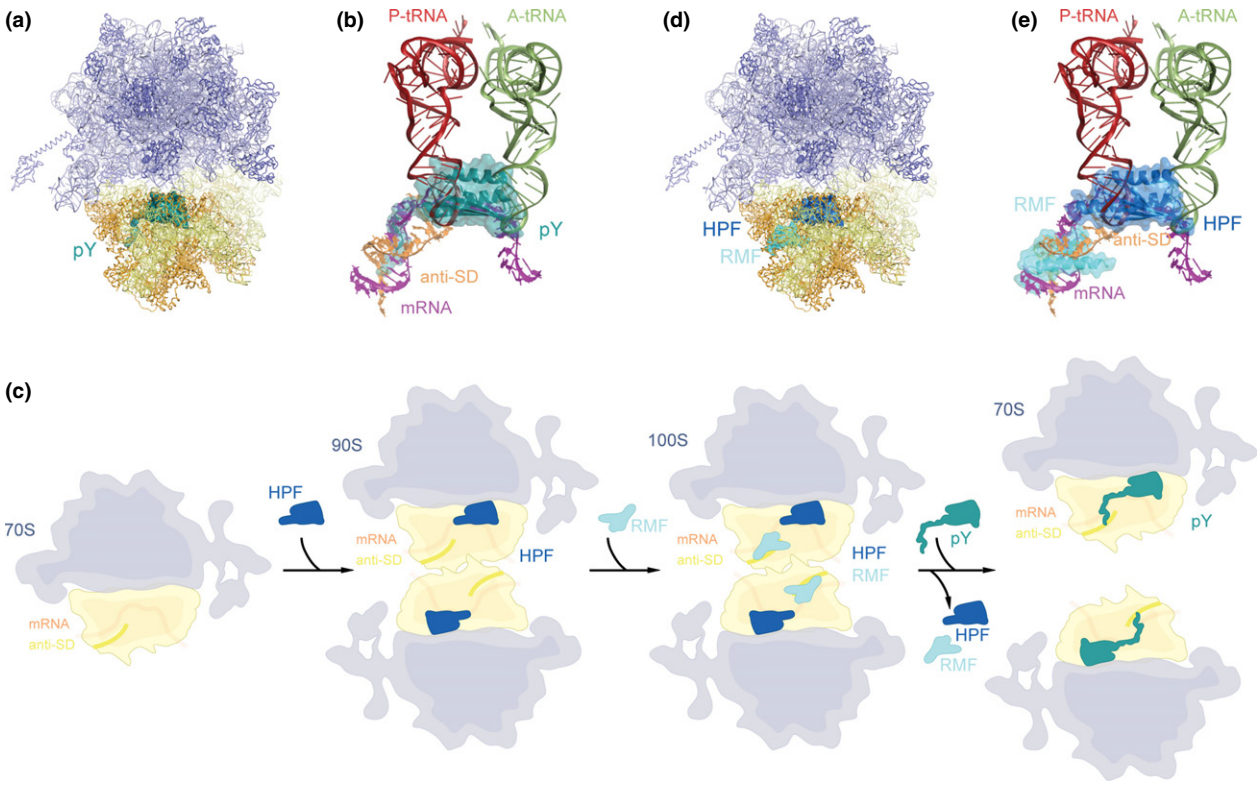

Fig. 7. Antagonistic action of pY and RMF/HPF on 100S formation. (a) Structure of pY on the 70S ribosome (Polikanov *et al.*, 2012). (b) Overlap in binding position of pY with mRNA and tRNAs in the A- and P-sites and CTD with RMF (Polikanov *et al.*, 2012). (c) Scheme for antagonistic action of pY to inhibit 100S formation and concerted action of RMF and HPF to promote 100S formation. (d) Structure of RMF and HPF on the 70S ribosome (Polikanov *et al.*, 2012). (e) Overlap in binding position of HPF with mRNA and tRNAs in the A- and P-sites and RMF with SD-antiSD region on the 30S subunit (Polikanov *et al.*, 2012).

dissociate into translationally active 70S ribosomes (Wada, 1998; Maki *et al.*, 2000). This process is rapid, reaching completion within 1 min (Aiso *et al.*, 2005). An *rmf* mutant does not form 100*S* ribosomes and loses viability in stationary phase earlier than the wild-type (Izutsu *et al.*, 2001a). Therefore, the 100S particles are thought to represent a protection and/or storage state, a phenomenon referred to as 'ribosome hibernation' (Yoshida *et al.*, 2002).

Crystal structures of RMF and HPF in complex with the 70*S* ribosome provide an explanation as to how these factors inactivate translation: HPF binds in a position overlapping tRNA and mRNA in the A- and P-site on the 30S subunit (Fig. 7d and e), analogously to pY, which is consistent with the high sequence identity (*c.* 40%) between HPF and pY. RMF also binds to the 30S subunit; however, the binding site overlaps with the Shine–Dalgarno (SD)-anti-SD helix (Fig. 7d and e). Collectively, these findings suggest that HPF and RMF could inhibit the initiation of protein synthesis by sterically preventing binding of initiator tRNA to the P-site as well as the association of the mRNA with the ribosome (Polikanov *et al.*, 2012). Electron microscopy studies revealed that 100S particles are comprised of two 70S ribosomes that dimerize via interaction between the 30S subunits (Wada, 1998; Yoshida *et al.*, 2002; Kato *et al.*, 2010; Ortiz *et al.*, 2010). A structural rationale for the RMF-induced dimerization of 70S ribosomes is proposed on the basis of the RMF-70S crystal structures, namely, that binding of RMF induces a conformational change in the head of the 30S subunit, resulting in a complementary surface that promotes interaction and dimerization with the 30S subunit of a second 70S ribosome (Polikanov *et al.*, 2012).

Despite the sequence similarity between pY and HPF, binding of pY is antagonistic to 100S formation and promotes 70S formation (Ueta *et al.*, 2005) (Fig. 7c). An explanation for this comes from the recent crystal structures revealing that the long C-terminal tail of pY, which is absent in HPF, is likely to extend through the mRNA channel into the SD-antiSD region and would prevent binding of RMF (compare Fig. 7a, b, d and e). Interestingly, 100S formation has recently been observed in bacteria that do not encode RMF (Ueta *et al.*, 2013). It appears that instead of RMF, these bacteria contain a long-form HPF/pY that is necessary and sufficient to induce 100S dimerization (Ueta *et al.*, 2013; Puri *et al.*, 2014). It is tempting to speculate that the additional CTD of the long-form HPFs, which is absent in the short-form *E. coli*-like HPFs, would function to induce 100S formation by inducing conformational changes in the head region of the small subunit, analogous to RMF.

The role of EttA and monitoring of ATP levels in the cell

Energy-dependent translational throttle A (EttA, Yjjk) is an ATP-binding cassette (ABC) protein of the ABC-F family (Boel *et al.*, 2014). Expression of EttA increases in stationary phase and the presence of the protein confers a fitness advantage to restarting growth when cells are transferred to fresh media (Boel *et al.*, 2014). In *E. coli*, EttA co-fractionates with both 70S ribosomes and polysomes, suggesting a role during translation (Boel *et al.*, 2014). Overexpression of an ATP-hydrolysis deficient EttA mutant (EttA-EQ_2), but not wildtype EttA, is detrimental to cell viability and leads to a decrease in polysomes relative to monosomes. Similarly, the addition of EttA-EQ_2 to *in vitro* translation assays results in inhibition of translation after formation of the first peptide bond (Boel *et al.*, 2014). Wildtype EttA slightly stimulates translation in the presence of ATP, however, inhibits translation when ADP is present – an effect that can be relieved by further increasing the ATP concentration. This suggests that an elevated ADP/ATP level, as found in energy-depleted cells (e.g. stationary phase), causes EttA to stabilize and hibernate an initiation state ribosome, until ATP levels are restored and EttA dissociates from the ribosome enabling translation to resume (Boel *et al.*, 2014) (Fig. 8a).

A cryo-EM structure of EttA-EQ_2 bound to a 70S ribosome initiation complex reveals that EttA binds in the E-site of the ribosome, sandwiched between ribosomal protein L1 and the P-site initiator tRNA (Fig. 8b and c) (Chen *et al.*, 2014). This is consistent with competition between EttA and binding of deacylated tRNA at the E-site of the ribosome (Chen *et al.*, 2014). The crystal structure of EttA reveals the presence of two tandem ABC domains separated by an *c.* 80 amino acid linker that forms a long α-helical extension (Boel *et al.*, 2014), which in the cryo-EM structure contacts the acceptor arm near to the CCA-end of the P-tRNA (Fig. 8c). However, additional structures, and at higher resolution, will be required to understanding how interaction of EttA-ATP with the P-tRNA stimulates peptide bond formation, whereas EttA-ADP interferes with the peptide-bond formation. Similarly, it remains unclear why EttA-ADP inhibits translation, whereas paradoxically the hydrolysis of EttA-ATP to EttA-ADP does not interfere with translation, but instead leads to release of EttA-ADP from the ribosome.

Other factors binding ribosomes under stationary phase stress

The stationary phase-induced ribosome-associated protein (SRA) binds tightly to 30*S* subunits and cannot be dissociated by high salt or low Mg^{2+} concentrations (Wada,

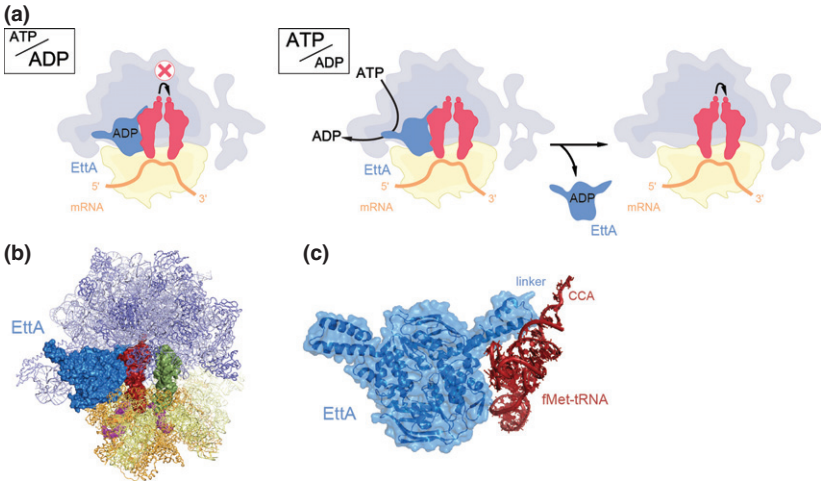

Fig. 8. Hibernation of translation initiation complex by EttA. (a) Scheme for the influence of the ATP/ADP ratio on EttA-mediated translation inhibition. (b) Structure of EttA on the 70S ribosome (Chen *et al.*, 2014). (c) EttA (blue) contacts the acceptor arm of the P-tRNA (Chen *et al.*, 2014).

1986). Because of this tight binding, SRA was thought to be a *bona fide* ribosomal protein and was formerly called S22 (*rpsV*). However, subsequent studies indicated that SRA binding is substoichiometric, being present as 0.1 copies per ribosomal particle in the exponential growth phase and increasing to *c.* 0.4 in stationary phase (Izutsu *et al.*, 2001b). The function of SRA is still unclear, as the deletion of *sra* affects neither 100*S* formation nor the survival of cells in the stationary phase. Like RMF, SRA is only found in enterobacteria, such as *E. coli* and *Salmonella typhimurium* (Izutsu *et al.*, 2001b).

The ribosome-silencing factor S (RsfS, previously referred to as RsfS, YbeB, DUF 143) is proposed to play a prominent role in the silencing of ribosome activity in stationary phase as well as during the transition from rich to poor media (Hauser *et al.*, 2012). RsfS is a conserved protein encoded by almost all bacterial and eukaryotic genomes, but not archaea. RsfS binds to the L14 protein of the 50S subunit and impairs subunit joining (Hauser *et al.*, 2012). RsfS helps cells to adapt to slow growth under restricted nutrient (poor media) and energy (stationary phase) conditions by down-regulating protein synthesis and thereby saving energy (Hauser *et al.*, 2012).

Recently, the hypothetical protein YqjD of *E. coli* was characterized as a membrane-integrated ribosome-binding protein (Yoshida *et al.*, 2012). YqjD is expressed during the stationary phase and expression is controlled by the stationary-phase sigma σ^S. YqjD is composed of a C-terminal transmembrane domain and an N-terminal domain which associates with 70*S* and 100*S* ribosomes. *Escherichia coli* possesses two paralogs to YqjD, namely ElaB and YgaM, which are expressed and bind to ribosomes in a similar manner to YqjD. Overexpression of YqjD causes inhibition of cell growth. It has been suggested that YqjD inhibits ribosomal activity and localizes ribosomes to the membrane during stationary phase (Yoshida *et al.*, 2012).

Hsp15 and the ribosomal heat shock response

Upon heat shock, a translating ribosome can dissociate erroneously (Korber *et al.*, 2000; Jiang *et al.*, 2009), resulting in a 50S subunit carrying a tRNA still attached to the nascent polypeptide chain (Fig. 9). Re-initiation of protein synthesis using these 50S subunits first requires the removal of the peptidyl-tRNA by hydrolysis of the ester linkage between the tRNA and the polypeptide chain (Jiang *et al.*, 2009). This function is thought to be carried out by Heat shock protein 15 (Hsp15) (Korber *et al.*, 2000; Jiang *et al.*, 2009). Hsp15 is widely distributed among bacteria and is encoded by *yrfH* in *E. coli.* In response to heat shock stress, *yrfH* is upregulated *c.* 50-fold, and therefore belongs within the top ten of highly expressed proteins (Richmond *et al.*, 1999). This upshift also exceeds the expression of other well-characterized proteins such as GroEL/ES, DnaK, ClpA and Lon, indicating its importance for adaptation to thermal stress (Richmond *et al.*, 1999; Wilson & Nierhaus, 2007; Jiang *et al.*, 2009). In the absence of Hsp15, the peptidyl-tRNA moiety is positioned at the A site of the PTC (Korber *et al.*, 2000; Jiang *et al.*, 2009). Binding of Hsp15 to this complex frees the A-site by stabilization the peptidyl-tRNA in the P-site (Fig. 9). Specifically, Hsp15 comprises an S4 RNA binding motif that interacts with helix 84 of the 23S rRNA located within the central protuberance of the 50S subunit (Jiang *et al.*, 2009), while the positively charged carboxy-terminal tail of Hsp15 contacts the elbow of the peptidyl-tRNA (Jiang *et al.*, 2009). These interactions appear to stabilize the peptidyl-tRNA at the P-site, such that it is optimally positioned to be hydrolyzed by the termination release factors, such as RF2 (Jiang *et al.*, 2009). A loop at the tip of domain three of RFs contains a conserved GGQ motif that is thought to participate in coordination of the water molecule neces-

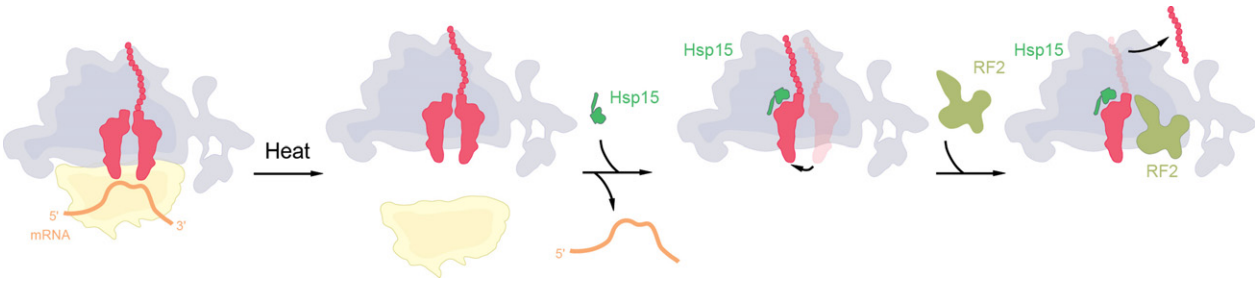

Fig. 9. Hsp15-mediated rescue of peptidyl-tRNA on 50S subunits. Scheme for the Hsp15-mediated translocation of peptidyl-tRNA from A- to P-site to allow peptidyl-tRNA hydrolysis and release of the polypeptide chain (possibly by recruitment of RF2) and subsequent recycling of the 50S subunits.

sary for nucleophilic attack and hydrolysis of the ester linkage between the CCA-end of the tRNA and the nascent polypeptide chain (Weixlbaumer *et al.*, 2008). Normally, RFs are recruited to ribosomes bearing stop codons in the A-site of the 30S subunit, however in Hsp15 rescued 50S complex, there is no stop codon (Jiang *et al.*, 2009). Whether Hsp15 is directly involved in recruitment of the RFs or whether another rescue factor participates, such as ArfB, remains unclear.

The ribosomal response to antibiotic stress

The ribosome is one of the major targets in the cell for antibiotics, and bacteria have developed a plethora of mechanisms by which to alleviate the stress associated with antibiotic inhibition and obtain resistance (Wilson, 2009, 2014). The majority of resistance mechanisms involve efflux of the drug from the cell or preventing interaction of the drug with the ribosome, either through modification or degradation of the drug or mutations/alterations within the drug binding site on the ribosome. However, protein factor-related mechanisms have also been discovered that alleviate antibiotic stress by binding to the ribosome: these include resistance to tetracycline by binding of the TetM-like proteins (Nguyen *et al.*, 2014) or fusidic acid resistance through the action of the FusB-like proteins (Farrell *et al.*, 2011).

Ribosome protection protein-mediated tetracycline resistance

Tetracycline binds to the 30S subunit and inhibits delivery of the aa-tRNA to the A-site (Brodersen *et al.*, 2000; Pioletti *et al.*, 2001). Resistance to tetracycline can be acquired by efflux pumps, mutations/deletions within the 16S rRNA, drug inactivation, or specialized ribosome protection proteins (RPPs) (reviewed by (Chopra & Roberts, 2001; Nguyen *et al.*, 2014). RPPs are ribosome-dependent GTPases displaying high homology with EF-G (reviewed by (Connell *et al.*, 2003a). Based on amino acid sequences, RPPs are divided into three groups: (1) *tetM*, *tetO*, *tetS*, *tetW*, *tet32*, *tet36*; (2) *tetBP*, *otrA*, *tet*; (3) *tetQ*, *tetT*. The *otrA* determinant, believed to be ancestor of RPPs, was found in the biosynthesis cluster of the oxytetracycline producer, *Streptomyces rimosus* (*otr* – oxytetracycline resistance gene) (Rawlings, 1999; Zhang *et al.*, 2006; Pickens & Tang, 2010). The *tetM* and *tetO* genes are the most prevalent, best characterized, and can confer resistance to tetracycline in both Gram-negative and Gram-positive bacteria (Nguyen *et al.*, 2014). The *tetM* determinant is predominantly associated with self-regulatory conjugative chromosomal elements, while the *tetO* gene can be found on conjugative plasmids or non-mobile, integrated into the chromosomal DNA (Chopra & Roberts, 2001).

RPPs confer resistance to tetracycline by binding to drug-inhibited ribosomes, displacing the drug and allowing delivery of ternary complex (Fig. 10a) (Connell *et al.*, 2003a; Nguyen *et al.*, 2014). Addition of purified TetO/TetM proteins into *in vitro* translation reaction, significantly increases the half-inhibitory concentration for tetracycline (Trieber *et al.*, 1998; Grossman *et al.*, 2012; Jenner *et al.*, 2013). TetM/TetO displace tetracycline in a GTP-dependent manner (Burdett, 1996; Trieber *et al.*, 1998); however, GTPase hydrolysis is necessary for turnover of RPPs rather than displacement of a drug, consistent with an observation that tetracycline release occurs in the presence of non-hydrolysable GTP analogs (Burdett, 1996; Trieber *et al.*, 1998). Cryo-EM reconstructions of *Enterococcus faecalis* and *Campylobacter jejuni* TetO bound to the *E. coli* 70S ribosome (Fig. 10b) confirmed that RPPs bind to the ribosome analogously to EF-G (Spahn *et al.*, 2001; Dönhöfer *et al.*, 2012; Li *et al.*, 2013). The more recent structures (Dönhöfer *et al.*, 2012; Li *et al.*, 2013) indicate that on the ribosome, RPPs directly encroach on the tetracycline-binding site and therefore most likely displace the drug from its binding site by direct mechanism, possibly via interaction with the nucle-

obase C1054 within h34 of the 16S rRNA (Fig. 10c) (Dönhöfer *et al.*, 2012). Specifically, two tyrosine residues (Y506 and Y507) within loop III of domain IV of TetM are proposed to alter the conformation of C1054 to perturb stacking interaction with tetracycline, leading to drug dissociation from the ribosome and preventing rebinding of the drug to the ribosome (Dönhöfer *et al.*, 2012). Based on footprinting studies, it has been proposed that this conformational change in C1054 induced by RPPs (in this case TetO) is retained by the ribosome after RPP dissociation, thus explaining why tetracycline does not rebind but rather allows or even promotes binding of the ternary complex EF-Tu-aa-tRNA-GTP (Connell *et al.*, 2003b). RPPs confer resistance to tetracycline, doxycycline, and minoxycycline; however, they are susceptible to the new classes of tetracyclines, such as the glycylcycline tigecycline (Nguyen *et al.*, 2014). It appears that the presence of bulky substitutions of tigecycline (that is absent in tetracycline) prevents loop III of the RPP from accessing C1054 (Fig. 10d) and thus the RPP cannot dislodge the glycylcycline (Dönhöfer *et al.*, 2012; Jenner *et al.*, 2013).

FusB-mediated fusidic acid resistance

In the presence of the antibiotic fusidic acid, elongation factor EF-G is locked on the ribosome (Fig. 10e) and the pool of actively translating ribosomes is diminished (Wilson, 2009). Fusidic acid interacts with the ribosome-bound conformation of EF-G and is thought to prevent conformational changes within EF-G that are necessary for dissociation from the ribosome (Wilson, 2009). Resistance to fusidic acid can be acquired by mutations within the *fusA* gene encoding EF-G, which either prevent binding of fusidic acid to EF-G or enable EF-G to undergo the conformational changes necessary for dissociation in

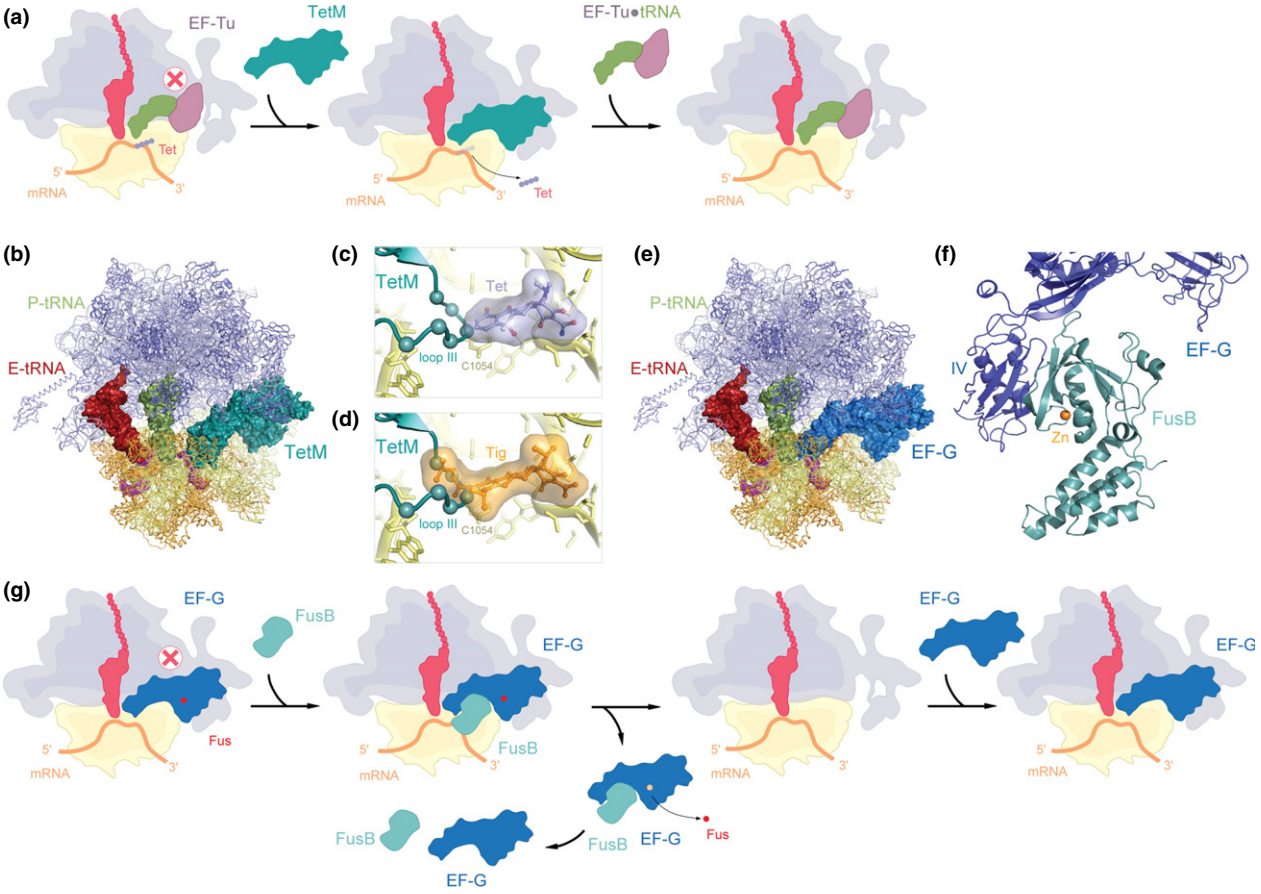

Fig. 10. Relief of antibiotic stress by TetM- and FusB-like proteins. (a) Scheme for TetM-mediated tetracycline resistance via ribosome binding and removal of tetracycline. (b) Structure of TetM on the 70S ribosome (Dönhöfer *et al.*, 2012). (c and d) Relative binding position of loop III of domain IV of TetM relative to (c) tetracycline and (d) tigecycline (Dönhöfer *et al.*, 2012; Jenner *et al.*, 2013). (e) Structure of EF-G stalled by fusidic acid on the 70S ribosome (Gao *et al.*, 2009). (f) Model for the interaction of FusB (teal) with domain IV of EF-G (blue) [based on (Cox *et al.*, 2012)]. (g) Scheme for FusB-mediated fusidic acid resistance via ribosome binding and dislodging the EF-G-fusidic acid complex.

the presence of the drug. Another common mechanism by which bacteria acquire fusidic acid resistance is through the horizontal transfer of *fusB*-like determinants (including *fusC* and *fusD*) (Farrell *et al.*, 2011).

The *fusB*-like genes are found in several Gram-positive bacteria including genera of *Listeria*, *Bacillus*, *Enterococcus*, *Lactococcus*, *Lactobacilli*, but are best characterized for *Staphylococcus* (O'Neill *et al.*, 2004; O'Neill & Chopra, 2006). *fusB* determinants are predominantly plasmid-encoded (Chopra, 1976; O'Brien *et al.*, 2002; O'Neill *et al.*, 2004; O'Neill & Chopra, 2006); however, they can also be integrated into chromosomal DNA of bacteria (O'Neill *et al.*, 2004). Expression of *fusB* gene is regulated through translational attenuation (O'Neill & Chopra, 2006): In the presence of fusidic acid, translation of the upstream encoded leader peptide results in ribosomal stalling. Stalling induces rearrangements of the secondary structure of mRNA exposing sequestered SD sequence and consequently allows translation of the downstream encoded *fusB* (O'Neill & Chopra, 2006).

FusB was shown to confer resistance to fusidic acid in *S. aureus*, both *in vitro* and *in vivo*; however it does not counteract the inhibitory effects of the drug on protein synthesis in *E. coli* (O'Neill & Chopra, 2006; Guo *et al.*, 2012). Consistently, biochemical experiments revealed that FusB interacts with *S. aureus* EF-G, but not with *E. coli* EF-G (O'Neill & Chopra, 2006; Cox *et al.*, 2012; Guo *et al.*, 2012). The presence of three additional amino acids (529-SNP-531) located in domain IV of *E. coli* EF-G but absent in *S. aureus* EF-G appears to be the reason for lack of interaction between FusB and *E. coli* EF-G (Cox *et al.*, 2013). Structures of isolated FusB and FusC reveal that this family of proteins contains two domains, an N-terminal four-helix bundle linked to a unique C-terminal α-β-fold domain that is stabilized by a zinc ion (Fig. 10f) (Cox *et al.*, 2012; Guo *et al.*, 2012). Currently, there are no available structures of FusB/C in complex with EF-G; however, NMR and mutagenesis studies indicate that the zinc-binding domain within the C-terminus of FusB contacts EF-G (Cox *et al.*, 2012, 2013). Similarly, biochemical assays analyzing the binding of FusB/C to truncated versions of EF-G, or to *E. coli*/*S. aureus* chimeric EF-Gs, suggest that domain IV of EF-G is the main determinant for FusB/C interaction (Cox *et al.*, 2012; Guo *et al.*, 2012) (Fig. 10f). The finding that the FusB/C binding site on EF-G is far away from the fusidic acid binding site would suggest that resistance is probably not conferred by sterically hindering the binding of fusidic acid to EF-G.

Steady-state and transient kinetics measurements using reconstituted *E. coli* components and *S. aureus* EF-G show that FusB binds to EF-G·GDP·FA stalled ribosomes, destabilizes the complex, and enhances the dissociation rate of EF-G (Cox *et al.*, 2012) (Fig. 10g). Presumably the destabilization of EF-G is due to space restrictions, as the interaction between the C-terminal domain of FusB/C with domain IV of EF-G cannot occur in the context of the canonical binding position of EF-G on the ribosome as seen in Fig. 10e. FusB also recognizes EF-G·GDP ribosome complexes in the absence of FA (Cox *et al.*, 2012), which suggests that FusB recognizes a distinct conformational state of EF-G on the ribosome, rather than the presence of the drug. In addition, it should be noted that FusB binds to free EF-G with a higher affinity than to ribosome-bound EF-G, suggesting the copy-number of FusB protein in the cell must be fine-tuned to the concentrations of the ribosome and EF-G (Cox *et al.*, 2012).

Other ribosome-associated stress factors

A number of ribosome-associated factors have been identified that appear to play important roles during stress, although their functions remain to be fully deciphered. Obg is an essential protein that appears to connect ribosome assembly with the nutrient status of the cell by interacting with SpoT and influencing ppGpp synthesis. While BipA and EF4 are not essential under optimal growth conditions, both factors confer a growth advantage to bacteria under specific stress conditions. Moreover, both factors appear to regulate translation of a specific and distinct subset of mRNAs, however, further investigation is required to fully understand their mechanism of action.

Obg and the link between ribosome biogenesis and the stringent response

The Obg family of proteins (also called ObgE, YhbZ, CgtA) are GTPases that are essential for viability in bacteria (Kint *et al.*, 2014). The *obg* gene was first described in *B. subtilis* as a part of spo**0B** operon, and called spo0B-associated **G**TP-binding protein (OBG) (Trach & Hoch, 1989). Homologs of Obg are found across all kingdoms of life and comprise five sub-families, namely Obg, Nog1, DRG, YchF and Ygr210 (Leipe *et al.*, 2002). Obg proteins have been linked to multiple functions ranging from cell cycle, DNA replication, and chromosome segregation to, sporulation and stress response; however, accumulating evidence indicates that Obg proteins may provide an important link between ribosome biogenesis and the stress response machinery (Kint *et al.*, 2014).

Bacterial Obg proteins have three domains, a central G domain flanked by unique N- and C-terminal domains, termed OBG and OCT (Obg-C-terminal), respectively (Fig. 11a) (Buglino *et al.*, 2002; Kukimoto-Niino *et al.*, 2004). Direct binding of Obg to 50S subunits has been

observed for bacterial Obg as well as homologs from yeast, namely, mitochondrial Mtg2p and nucleolar Nog1p (Wilson & Nierhaus, 2007). Depletion of Obg from the cell leads to a decrease in 70S ribosomes, an increase in both 30S and 50S subunits, as well as the appearance of an intermediate pre-50S particle (Sato *et al.*, 2005; Jiang *et al.*, 2006). The precursors to both the 16S and 23S rRNA are significantly increased, suggesting that RNA processing is impaired (Jiang *et al.*, 2006). Analysis of the pre-50S particle reveals reduced levels of r-proteins L33, L34 and L16 (Jiang *et al.*, 2006). These three proteins are late assembly proteins, suggesting that Obg is involved in a late step in 50S biogenesis. Mutagenesis studies suggest that the interaction of Obg with the ribosome is facilitated by the N-terminus of Obg (Kobayashi *et al.*, 2001; Datta *et al.*, 2004; Jiang *et al.*, 2006; Kuo *et al.*, 2008).

Moreover, Obg can bind ppGpp in the active site with an affinity similar to GDP (Buglino *et al.*, 2002; Persky *et al.*, 2009). High concentrations of (p)ppGpp abolish interaction of Obg with the 50S subunit (Jiang *et al.*, 2006). Two-hybrid and pull-down assays have shown that Obg interacts directly with SpoT, an enzyme that degrades (p)ppGpp (Wout *et al.*, 2004; Raskin *et al.*, 2007). Consistently, the depletion of *obg* in *E. coli* and *V. cholerae* leads to elevated levels of (p)ppGpp and induction of the global stress response (Jiang *et al.*, 2007; Raskin *et al.*, 2007). However, during amino acids starvation, the absence of *obg* does not alter levels of (p)ppGpp (Jiang *et al.*, 2006). In *V. cholerae*, Obg is no longer essential in the *relA*$^-$ background, indicating that the essentiality of Obg is linked to its ability to regulate ppGpp levels (Raskin *et al.*, 2007). Collectively, these findings suggest that Obg is a negative regulator of the stringent response via stimulation of SpoT activity and that the Obg-SpoT interaction is necessary to prevent an inappropriate activation of the stress response under nutrient-rich conditions (Jiang *et al.*, 2007; Raskin *et al.*, 2007).

EF4, a back-translocase that operates under stress

EF4 (LepA) is a paralog of an elongation factor EF-G and belongs to a superfamily of ribosome-dependent GTPases (Leipe *et al.*, 2002; Margus *et al.*, 2007). The *lepA* gene was discovered as the first ORF of the biocistronic *lep* operon of the **le**ader **p**eptidase I (*lepB*) in *E. coli* (Date & Wickner, 1981; March & Inouye, 1985). With the exception of *Streptococcus pyogenes* strain MGAS8232 and the endosymbiont *Carsonella ruddii*, the *lep* gene is ubiquitously conserved among bacteria as well as in mitochon-

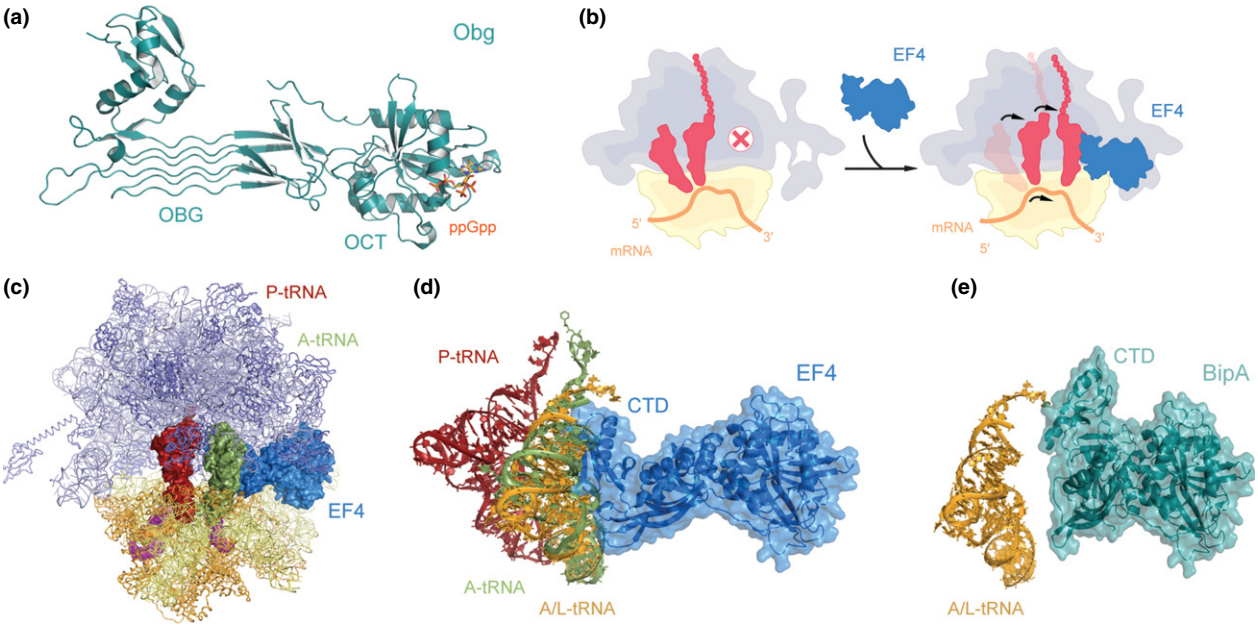

Fig. 11. Ribosome-associated stress factors Obg, EF4 and BipA. (a) Structure of Obg, with N-terminal OBG and C-terminal OCT domain flanking the G domain bound with ppGpp in the active site (Buglino *et al.*, 2002; Kukimoto-Niino *et al.*, 2004). (b) Scheme for EF4-mediated back-translocation of tRNAs on the ribosome. (c) Structure of EF4 bound to the 70S ribosome (Connell *et al.*, 2008). (d) Interaction of EF4 (blue) with distorted A/L-tRNA (orange), relative to canonical P-tRNA (green) and E-tRNA (red) (Connell *et al.*, 2008). (e) Structure of BipA (teal, PDB3E3X) relative to distorted A/L-tRNA from EF4-70S complex (Connell *et al.*, 2008) (aligned to EF4 based on the conserved G domain).

dria and chloroplasts (Leipe *et al.*, 2002; Margus *et al.*, 2007). Despite the high conservation, which implies an important role for EF4, deletion of the *lepA* gene has little or no effects under laboratory conditions on the viability of *E. coli* (Dibb & Wolfe, 1986), *S. aureus* (Colca *et al.*, 2003), and *Streptomyces coelicolor* (Badu-Nkansah & Sello, 2010). Similarly, the absence of chloroplast EF4 (cpEF4) in *Arabidopsis thaliana* (Ji *et al.*, 2012) and mitochondrial EF4 (Guf1) in *Saccharomyces cerevisiae* (Bauerschmitt *et al.*, 2008) has only mild phenotypes. Nevertheless, under specific conditions, EF4 can be essential for survival, for example *Helicobacter pylori* strictly requires EF4 to sustain growth at acidic pH (Bijlsma *et al.*, 2000).

EF4 associates with membranes (March, 1992; Pech *et al.*, 2011; Ji *et al.*, 2012) and was shown to play important role in the fidelity of mitochondrial protein synthesis (Bauerschmitt *et al.*, 2008) as well as chloroplast biogenesis (Ji *et al.*, 2012). Deletion of *lepA* leads to hypersensitivity to potassium tellurite, penicillin G (Shoji *et al.*, 2010), and high Mg^{2+} ion concentrations (Pech *et al.*, 2011). EF4 is present in *E. coli* at *c.* 0.06 copies per ribosome (Pech *et al.*, 2011). Moreover, the copy number of EF4 increases 2–3 times during stress conditions of high ionic strength or low pH, suggesting a role in maintaining growth under unfavorable conditions (Pech *et al.*, 2011). The deletion of *lepA* does not affect missense, non-sense, or frameshift errors, indicating that the fidelity of translation *in vivo* does not require EF4 (Shoji *et al.*, 2010). Similarly, EF4 did not affect accuracy of poly(U)-dependent poly(Phe) synthesis *in vitro* at low and high magnesium conditions (Pech *et al.*, 2011). High magnesium conditions are detrimental to translation and cause ribosome stalling, thus leading to the proposal that the function of EF4 is re-mobilize the stalled ribosomes (Yamamoto *et al.*, 2014).

EF4 shares sequence and structural homology with domains I, II, III, and V of EF-G (Qin *et al.*, 2006; Evans *et al.*, 2008), whereas the equivalent to domain IV of EF-G is replaced in EF4 with a unique C-terminal domain comprising one long α-helix cradled by four short strands of β-sheet (Evans *et al.*, 2008). Like EF-G, EF4 binds to ribosomes *in vivo* (Colca *et al.*, 2003) and *in vitro* (Qin *et al.*, 2006), and displays ribosome-dependent GTPase activity (Qin *et al.*, 2006; Mikolajka *et al.*, 2011) which is inhibited by thiostrepton (Starosta *et al.*, 2009; Mikolajka *et al.*, 2011; Walter *et al.*, 2012). However, biochemical data indicate that, unlike EF-G which promotes translocation of tRNAs, EF4 facilitates back-translocation of tRNAs (Qin *et al.*, 2006; Liu *et al.*, 2010a, b) (Fig. 11b). Recently, fast kinetic studies showed that EF4 strongly competes with EF-G for interaction with PRE complex, suggesting that the pre- rather than the post-translocational state is the physiological substrate for EF4 (Liu *et al.*, 2011). A cryo-EM structure of EF4 bound to a post-translocational state ribosome revealed that back-translocation had occurred such that the tRNAs were present in the A- and P-sites (Fig. 11c) (Connell *et al.*, 2008). Interestingly, the tRNA in the A-site was observed to adopt a distorted conformation due to interaction with the unique C-terminal domain of EF4, such that the acceptor arm of the A-tRNA is shifted away from the PTC (Fig. 11d). High magnesium conditions have been proposed to inhibit translation by inducing non-productive translocation of tRNAs during elongation. In this context, EF4 has been proposed to rescue these stalled ribosomes by catalyzing back-translocation and thus providing EF-G another opportunity to catalyze a productive translocation reaction to allow translation to continue (Yamamoto *et al.*, 2014).

BipA and translation regulation of virulence genes

BipA (TypA) is a paralog of EF-G and belongs to the superfamily of translational GTPases (Leipe *et al.*, 2002; Margus *et al.*, 2007). BipA is highly conserved among bacteria and in chloroplasts but, however, is absent in organisms with reduced genomes (Leipe *et al.*, 2002; Margus *et al.*, 2007). Although the exact molecular role of BipA is poorly understood, BipA was suggested to be involved in regulation of several cellular processes. BipA was described for the first time in *Salmonella typhimurium* as a protein involved in resistance to bactericidal/permeability-inducing protein (BPI) – a cathionic antimicrobial peptide produced by human neutrophils, and therefore was called **B**PI-**i**nducible **p**rotein **A** (BipA) (Qi *et al.*, 1995).

BipA lacks the region corresponding to domain IV of EF-G but has a distinct C-terminal domain (Fig. 11e) that is essential for interaction with ribosome and probably defines the role of BipA during translation (deLivron *et al.*, 2009). BipA exhibits ribosome-dependent GTPase activity (Farris *et al.*, 1998; deLivron *et al.*, 2009; Mikolajka *et al.*, 2011), which, like EF-G and EF4, can be inhibited by thiostrepton (Starosta *et al.*, 2009; Mikolajka *et al.*, 2011). *In vitro*, BipA, associates with 70S ribosomes in the presence of the non-hydrolysable GTP analog GDPNP, but binds to 30S subunit in the presence of ppGpp (deLivron & Robinson, 2008). *In vivo*, BipA co-sediments with 70S ribosomes under normal growth conditions, while during amino acid starvation, cold- or heat-shock, BipA is found on the 30S subunit (deLivron & Robinson, 2008). BipA of entheropathogenic *E. coli* (EPEC) undergoes tyrosine phosphorylation and was therefore named **Ty**rosine **p**hosphorylated protein A

(TypA). Phosphorylation of BipA enhances the GTPase activity *in vitro* (Farris *et al.*, 1998).

BipA in not essential for growth under optimal conditions (Farris *et al.*, 1998; Rowe *et al.*, 2000; Pfennig & Flower, 2001; Kiss *et al.*, 2004; Neidig *et al.*, 2013); however, it is required for growth at low temperatures (Pfennig & Flower, 2001; Kiss *et al.*, 2004). BipA regulates virulence and stress response in pathogenic [*E. coli* (EPEC), *P. aeruginosa*, *S. typhimurium*] and non-pathogenic bacteria (Farris *et al.*, 1998; Grant *et al.*, 2003; de-Livron & Robinson, 2008; Hansen *et al.*, 2013; Neidig *et al.*, 2013), as well as bacterial resistance to host antimicrobial peptides (Farris *et al.*, 1998), antibiotics, low pH, oxidative or detergent stress (Qi *et al.*, 1995; Barker *et al.*, 2000; Kiss *et al.*, 2004; Duo *et al.*, 2008; Neidig *et al.*, 2013). BipA is important for biofilm formation (Overhage *et al.*, 2007), flagellum-mediated motility (negatively) (Farris *et al.*, 1998; Grant *et al.*, 2003; Overhage *et al.*, 2007) and expression of K5 capsule genes in *E. coli* (Rowe *et al.*, 2000).

The lack of BipA does not influence the fidelity of translation (Shoji *et al.*, 2010). BipA, like EF4, can inhibit tmRNA tagging, suggesting its role as an elongation factor; however, unlike EF4, BipA does not prevent A-site mRNA cleavage (Shoji *et al.*, 2010). Deletion of a gene-encoding pseudouridine synthase *rluC* or substitution of 23S rRNA nucleotides 955, 2504 or 2580 suppresses the cold-sensitivity phenotype and alters expression of K5 capsule genes (Krishnan & Flower, 2008), suggesting that BipA acts as a translation factor rather than a modulator of transcription.

Outlook

The rapid advances in deep-sequencing techniques, coupled with the ribosome-profiling methodologies, have enabled researchers to obtain global overviews of ribosomal positions and occupancies on all cellular mRNAs in bacteria such as *E. coli* and *B. subtilis* (Oh *et al.*, 2011; Li *et al.*, 2012). Moreover, recent developments employing selective ribosome profiling has enabled global monitoring of factor-specific interactions with translating ribosomes, as demonstrated for the bacterial trigger factor chaperone (Oh *et al.*, 2011). Employing such methodologies to investigate the stress response factors outlined in this review will provide much needed insight into the specific functional states that these factors recognize. Similarly, ribosome profiling has been performed for bacteria grown under different stress conditions, such as *E. coli* grown in different media (Li *et al.*, 2012), revealing an integrated picture of how the translational stress response changes at a global level. Ultimately, time-resolved selective ribosome profiling under different stress conditions will yield a wealth of information about the molecular trigger leading to translational stress response, which in turn modulates the metabolic and morphological state of the cell.

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