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REVIEW ARTICLE

The paradox of elongation factor 4: highly conserved, yet of no physiological significance?

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LepA [EF4 (elongation factor 4)] is a highly conserved protein found in nearly all known genomes. EF4 triggers back-translocation of the elongating ribosome, causing the translation machinery to move one codon backwards along the mRNA. Knockout of the corresponding gene in various bacteria results in different phenotypes; however, the physiological function of the factor *in vivo* is unclear. Although functional research on Guf1 (GTPase of unknown function 1), the eukaryotic homologue of EF4, showed that it plays a critical role under suboptimal translation conditions *in vivo*, its detailed mechanism has yet to be identified. In the present review we briefly cover recent advances in our understanding of EF4, including *in vitro* structural and biochemical studies, and research on its physiological role *in vivo*. Lastly, we present a hypothesis for back-translocation and discuss the directions future EF4 research should focus on.

Key words: back-translocation, elongation factor 4 (EF4) (LepA), GTPase of unknown function 1 (Guf1), mitochondrion.

INTRODUCTION

The ribosome is a universally conserved ribonucleoprotein machine present in all living cells which uses aa-tRNA (aminoacyl-tRNA) substrates to translate genomic information encoded in an mRNA [1–5]. Translation can be roughly divided into four stages: initiation, elongation, termination of protein synthesis and recycling of the ribosome [6–10]. In each of these phases, additional protein factors are required, many of which are ribosome-dependent GTPases classified as trGTPases (translational GTPases) [11–14].

As the elongation cycle is the stage where the new polypeptide is assembled, it may be regarded as the major component of protein synthesis [6,12]. During each cycle, one amino acid is added to the growing polypeptide chain. This process is a repetitive multistep process which includes decoding, peptidyl transfer and translocation of mRNA•2tRNAs, and is governed by two universal EFs (elongation factors), termed EF-Tu and EF-G in bacteria (Figure 1), and eEF (eukaryotic EF) 1α and eEF2 in archaea and eukaryotes [15–17]. At the beginning of each elongation cycle, the ribosome is in a state known as the POST (post-translocational) state, where the E site is occupied by a deacylated tRNA, the P site is occupied by a peptidyl tRNA and the A site remains empty. EF-Tu delivers aa-tRNA to the POST ribosome in a ternary complex of EF-Tu•GTP•aa-tRNA, and plays an important role in ensuring the fidelity of decoding (Figure 1) [18]. The decoding process occurs at this point: the anti-codon of a cognate aa-tRNA recognizes the codon at the A-site mRNA [12]. Thereafter, EF-Tu hydrolyses GTP and leaves the ribosome with GDP [16]. Accommodating a cognate aa-tRNA fully into the A site leads to a rapid peptidyl transfer reaction, thus deacetylating P-tRNA and transferring the nascent polypeptide to the A-site tRNA to form the PRE (pre-translocational) ribosomal complex (Figure 1). This process is catalysed by the rRNA of the 50S subunit [19–22]. The third step in the elongation cycle is translocation, catalysed by EF-G•GTP [23]. Peptidyl-tRNA and deacylated tRNA are translocated from the A- and P-sites to the P- and E-sites respectively (Figure 1). Translocation of these two tRNAs is concomitant with movement of the mRNA by one codon length [24–32]. The following codon thus enters the A site to wait for a new aa-tRNA, and the cycle continues until a stop codon (UAA, UAG or UGA) enters the A site (Figure 1) [33–35]. In higher fungi, such as yeast and *Candida albicans*, a third EF, EF3, has been identified as an essential factor for the peptide elongation step, and also as a protein indispensable for yeast viability [36–38]. EF3 is an ATP-dependent E site factor required for opening the E site and thus releasing the E site deacylated tRNA upon accommodation of the A site aa-tRNA [17,36,39]. Furthermore, EF3 and ATP were hypothesized to catalyse the post-termination complex splitting it into ribosome, mRNA and tRNA, which need to be recycled for the next round of translation [40,41].

A fourth EF, EF4, has been identified as a *bona fide* trGTPase in bacteria. It catalyses back-translocation of the P- and E-tRNAs to the A- and P-tRNAs [42–44]. It was originally called LepA [45], since the gene encoding the protein is the first cistron of the bicistronic *lep* operon of leader peptidases (LepB or Lep) in *Escherichia coli* [46]. EF4 has been shown to bind the ribosome *in vivo* as well as *in vitro* [47]. Intriguingly, even though EF4 is highly conserved among all bacteria and nearly all eukaryotes, very little is known about its physiological role *in vivo*.

STRUCTURE OF EF4

E. coli EF4 is a protein containing 599 amino acid residues with a molecular mass of 67 kDa. Using X-ray crystallography, Steitz and co-workers determined the crystal structure of EF4 from *E. coli* at a 2.8 Å (1 Å = 0.1 nm) resolution (PDB code 3CB4)

Abbreviations used: aa-tRNA, aminoacyl-tRNA; cryo-EM, cryo-electron microscopy; CTD, C-terminal domain; eEF, eukaryotic elongation factor; EF, elongation factor; 4E-BP1, eukaryotic initiation factor 4E-binding protein 1; GAC, GTPase-associated centre; Guf1, GTPase of unknown function 1; IF, initiation factor; POST, post-translocational; PPIase, peptidylprolyl *cis–trans* isomerase; PRE, pretranslocational; trGTPase, translational GTPase; S6K1, S6 kinase 1; TOR, target of rapamycin.

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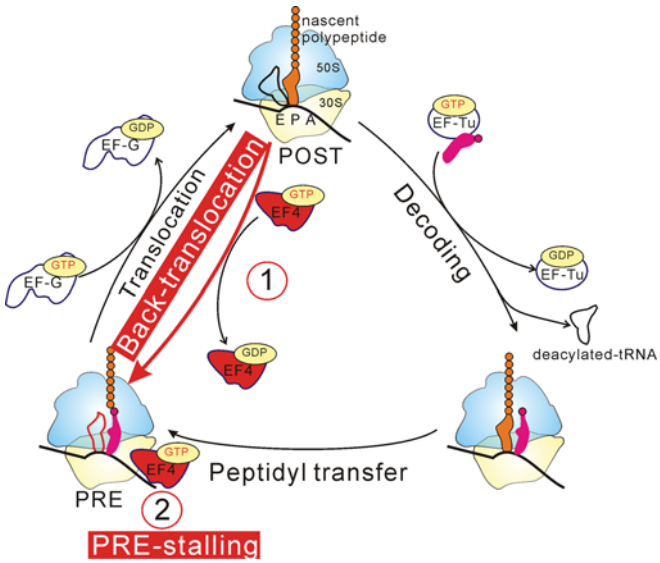

Figure 1 The elongation cycle of bacterial protein synthesis and the role of EF4

Each cycle of translation elongation is composed of three sequential steps: (i) decoding; (ii) peptidyl transfer; and (iii) translocation of mRNA•2tRNAs. (i) The decoding step is catalysed by EF-Tu (eEF1α in eukaryotes). EF-Tu•GTP brings aa-tRNA (pink) into the ribosome. After decoding, cognate aa-tRNA will be accommodated into the A site and EF-Tu will hydrolyse GTP to GDP. With lower affinity, EF-Tu•GDP and deacylated tRNA (unfilled black-edged) will be released from the ribosome. (ii) Peptidyl transfer is the step where the peptide on P-tRNA (orange) is transferred on to A-tRNA (pink). This reaction is catalysed by 50S rRNA. Thus P-tRNA is deacylated (unfilled orange) and A-tRNA possesses the polypeptide elongated by one residue (pink bead). (iii) Translocation is triggered by GTP-bound EF-G (eEF2 in eukaryotes). The mRNA•2tRNAs complex will be translocated from A and P sites (PRE state) to P and E sites (POST state). With the task accomplished, EF-G•GDP will leave the ribosome. EF4 targets both the PRE- and POST-state ribosome. EF4 (red) targets (1) the POST complex and catalyses the back-translocation of mRNA•2tRNAs to the PRE state, and (2) the PRE-stalling ribosome.

[48]. The structure of EF4 is very similar to EF-G. EF4 can be subdivided into five domains. Domains I, II, III and V of EF-G are found in EF4, but there is no counterpart of domain IV and G'. However, EF4 contains a unique CTD (C-terminal domain) that is not found in any other trGTPase [43,48,49] (Figures 2A and 2B).

Domain I (also called the G domain) and domain II are conserved in both structure and sequence in all trGTPases [49,50], suggesting that these two domains work together in the ribosome [28,51]. The G domain is a common building block responsible for GDP/GTP binding in all G-binding proteins [52]. It interacts with ribosomal proteins L7/L12 [53,54] and L11 [55] in the GAC (GTPase-associated centre) of the 50S large ribosomal subunit. Domain II is the contact site of a trGTPase to a small rRNA 16S shoulder region [56,57]. Domains III and V contain a common motif, referred to as a double-split β-α-β motif, observed in many other RNA-binding proteins and R-proteins responsible for RNA binding [58–61]. Domain III contacts the body region of 30S small ribosomal subunit and domain V contacts helices 43 and 44 of 23S rRNA in the GAC of 50S subunit [62,63]. Remarkably, there is a common feature to all of these four mutual domains: they exclusively contact the ribosome, but not, however, the molecule being translocated, i.e. mRNA•2tRNAs (Figure 2B).

The modelled part of the CTD is composed of one long α-helix that is cradled by four short strands of the β-sheet. In contrast, the structure of the last 44 residues of the CTD could not be defined in the X-ray structure [48]. EF4-CTD is universally conserved among different EF4 homologues, and can be regarded as an identity element of this protein, i.e. if a protein contains an EF4-CTD, it is classified as EF4 [49]. The CTD contains a number of positively charged residues of lysine or arginine (27 in *E. coli*) and under physiological conditions it has a predicted net charge of approximately +13 [49,62]. This is indicative of its direct interaction with the A site pepidyl-tRNA. However, little is known about the function of the CTD. A cryo-EM (cryo-electron microscopy) study of the ribosomal complex containing EF4 and tRNAs provides some clues as to what the function of these conserved lysine/arginine residues in the CTD [62] might be, as discussed below.

THE MOLECULAR ROLE OF EF4 IN PROTEIN SYNTHESIS

EF4 is conserved in almost all known genomes

EF4 is one of the most conserved proteins in bacteria, and its amino acid identity ranges from 55 % to 68 % between bacterial orthologues. It is even more conserved than other essential bacterial translation factors, such as IF (initiation factor) 3 (43–69 %) and guanine-exchange factor EF-Ts (33–50 %) [43]. EF4 is not found in archaea or in the cytoplasm of eukaryotes, but, with three exceptions, one copy of the *lepA* gene is present in all bacteria and mitochondria/chloroplasts. The three exceptions are: (i) *Streptococcus pyogenes* MGAS8232 NC 003485 has no *lepA* gene, whereas four other *Streptococci pyogenes* as well as other *Streptococci* strains do have a *lepA* gene; (ii) *Carsonella ruddii*, an obligate symbiont which has a very small genome and lives in lice [64], contains no *lepA* gene; and (iii) *Pirellula*, which has two copies of the *lepA* gene [49,65,66].

EF4 catalyses back-translocation

The involvement of EF4 in translation was found in a photoactivatable assay where the antibiotic oxazolidone was linked to EF4 and the PTC (peptidyl transferase centre) of the ribosome at the same time [47]. Cryo-EM reconstructions of the EF4-bound ribosome complex showed that the G domain of EF4 interacted with the same region of the ribosome as other trGTPases [EF-Tu, EF-G, RF3 (release factor 3) or IF2], namely the GAC [62]. An NMR study revealed that interactions of EF4 with the ribosomal proteins are similar to those of EF-G [54]. This similarity has been confirmed by uncoupled ribosome-dependent GTPase activity analysis [42,43]. EF4 displays an activity as strong as that of EF-G, and much stronger than that of other trGTPases [43,67]. TrGTPases function as molecular switches in several processes of cellular regulation by cycling between the GTP state (active) and the GDP state (inactive) [68]. Similar to EF-G, the substitution of GDP with GTP in EF4 is independent of guanine-exchange factors, since EF4 has a higher affinity for GTP than for GDP [69].

The most remarkable characteristic of EF4 is its ability to catalyse the backward movement of peptidyl-tRNA and deacylated tRNA in a direction opposite to that catalysed by EF-G, i.e. from the P and E sites to the A and P sites (Figure 1). Hence, EF4 is regarded as a back-translocase, as opposed to (forward) translocases, i.e. EF-G. This function of EF4 was determined using puromycin reaction, dipeptide formation and toeprinting assays [43]. Structural details were obtained in the cryo-EM study [62] (Figure 2B). Interestingly, after the discovery of EF4, it was reported that the back-translocation of P and E tRNAs to A and P tRNAs can occur spontaneously at a very slow rate provided that: (i) the E site contains a tRNA; and (ii) EF-G is not associated with the ribosome [70,71].

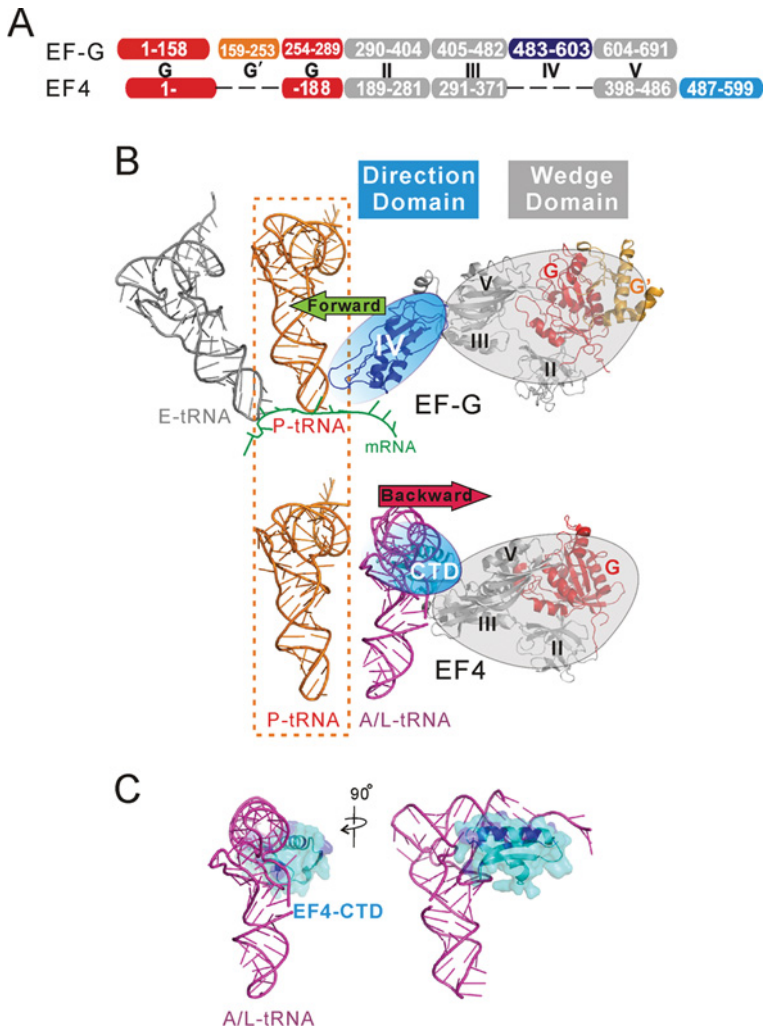

Figure 2 Sequence and structural comparisons of EF4 with EF-G

(**A**) Comparison of the domain structures of EF4 and its orthologues with EF-G. Red, G domain; orange, G' domain; grey, the domains of EF-G (II, III and V) which are conserved in EF4; blue, domain IV of EF-G (the pawl in the Brownian ratchet); cyan, EF4-CTD. Broken line: EF-G residues missed in EF4. (**B**) Comparison of the interaction of EF-G with tRNAs in the POST complex (PDB code 2WRI) and that of EF4 with tRNAs in the PRE (A/L) complex (PDB code 3DEG). The mutual domains of EF-G and EF4, namely domains I, II, III and V, compose the 'wedge domain' (grey area) that only contacts the ribosome. The factor-specific domain, namely domain IV of EF-G or EF4-CTD, is the 'direction domain' (blue area) that contacts peptidyl-tRNA (and mRNA) and is responsible for forward (green arrow) or backward (red arrow) translocation respectively. (**C**) EF4-CTD interacts with the anti-codon stem loop (ASL) of A/L tRNA. The dark blue residues in EF4-CTD are the conserved positively charged residues that contribute most to electrostatic interactions with the ASL.

Despite the large amount of structural and biochemical data, the mechanism by which EF-G catalyses forward translocation of tRNAs on the ribosome has not been completely elucidated. A Brownian Ratchet Machine model has been proposed to describe the motion characteristics of an elongating ribosome along an mRNA and the function of EF-G as the determinant for direction [1,72,73]. A Brownian Ratchet Machine refers to a molecular scale device that can perform work by rectifying its thermal motions to drive it forwards or backwards with equal probability [74]. In this model, after the peptidyl transfer has occurred, the peptidyl and deacylated tRNAs are in a thermodynamic equilibrium between the PRE and POST state, and EF-G functions as the determinant for the forward movement of tRNAs, the pawl of a Brownian Ratchet [1,72,73]. For this, domain IV of EF-G in the EF-G•GDP state would prevent tRNA back-translocation, thus functioning as a 'door-stop'. Analogous to EF-G, EF4 promotes a ratchet-like movement of ribosomal subunits [62,75]. The lack of the EF-G domain IV in the EF4 structure allows a peptidyl-tRNA to occupy the A site. Conserved positively charged residues in the CTD may actively draw the tRNA back by electrostatic interaction. Thus EF4 stabilizes a novel intermediate state of the A-tRNA in the A/L position (L stands for LepA and represents a new conformation of the acceptor arm of A-tRNA) [62] (Figure 2C). According to the Brownian Ratchet model, EF4 may function as a second pawl, providing directional information opposite to that of EF-G.

It is worth noting that only the factor-specific domain, namely domain IV of EF-G or the CTD of EF4, contacts the molecular complex being translocated (Figure 2B). In contrast, the mutual domains of the two factors contact only the ribosome. Since both factors can promote the ratcheting, we hypothesize that it is the mutual domains that are responsible for this activity. Ratcheting is the precondition of mRNA•2tRNAs translocation. It refers to the ratcheting-like rotation of the small ribosomal subunit relative to the large subunit, hence to loosen the connections between the two subunits [76]. Similar to a wedge, the four mutual domains function as one unit, maintaining the distance between the two ribosomal subunits so as to stabilize the ratcheted state. Hence

we suggest calling this unit the 'wedge domain' (Figure 2B). The second functional unit of the two translocases plays its role in determining the direction of translocation, therefore it can be regarded as the 'direction domain' (Figure 2B). The forward domain, i.e. domain IV of EF-G, through direct contact with the codon–anticodon bases of mRNA•peptidyl-tRNA, prevents the backwards movement of the complex (Figure 2B, top panel). In contrast, the backward domain, i.e. EF4-CTD, draws back peptidyl-tRNA and its associated mRNA•2tRNAs complex through positively charged residues (Figure 2B, bottom panel and Figure 2C).

A detailed analysis of the kinetic mechanism of EF4-catalysed back translocation reveals that movement of the mRNA•2tRNA from the POST to the PRE (L) (L refers to LepA) complex occurs via three intermediate steps: POST→I1→I2→I3→PRE [77], where I1–I3 represent the intermediates 1–3. Similar to EF-G-catalysed translocation, EF4-catalysed back-translocation requires the binding of GTP or its non-hydrolysable analogue GDPNP [77,78]. Initially, binding of EF4•GTP stabilizes the ratcheted state of the ribosome, which is associated with the transfer of two tRNAs from the classical E/E, P/P state to the hybrid P/E, A/P state. The factor is then able to further draw tRNAs into a classical A/A or even further into an A/L state by electrostatic attraction between the conserved lysine/arginine residues in EF4-CTD and bases in the acceptor arm of peptidyl-tRNA [48,62,75,77]. GTP hydrolysis is not necessary for back-translocation, since EF4•GDPNP can induce complete back-translocation as observed in the cryo-EM study [62]. Remarkably, in either spontaneous back-translocation or EF4-catalysed back-translocation, an E-tRNA is required [43,70]. These observations emphasize another physiological significance of E-tRNA: upon back-translocation, the P site will be re-occupied by deacylated tRNA from the E site and 30S subunit P site is therefore maintained by a tRNA, which is a prerequisite for the ribosome to maintain the correct reading frame in all intermediate states of translocation [6].

EF4•GTP binds not only to the POST complex, but also to the PRE complex, competing with EF-G to inhibit the elongation cycle [79,80] (Figure 1). The rate of the reaction of EF4 with the PRE complex is as rapid as that of EF-G with the PRE complex [80]. Such effects of EF4 would be expected to slow down peptide synthesis and thereby facilitate co-translational folding of nascent polypeptide [79–81]. *In vitro* and *in vivo* studies [82–85], as well as bioinformatic analyses of codon usage [86,87], provide strong evidence that a transient pause of polypeptide elongation can increase the fraction of active protein. Such short breaks may facilitate co-translational folding. Thus the blocking effect of EF4 on the PRE complex may play an important role in co-translational folding, especially under ionic stress, e.g. high concentration of magnesium or telluride [81,88,89]. In addition, we found that EF4, as well as other universal trGTPases, possess folding enzyme characteristics, which could be related to an ancient function of this protein family in the translation process [54,55]. A detailed discussion will be provided in the section below.

Overexpression of EF4 has been found to be toxic to the *E. coli* cell [43]. It is understandable that too much of such back-movement or stalling would harm the translation process. As a matter of fact, Nature had to ensure that EF4 is tightly controlled. There are numerous rare codons in the open reading frame of EF4, suggesting that EF4 is normally expressed at a low level compared with the very abundant EF-G and EF-Tu [57,67]. Even if EF4 could compete with EF-G to interact with the PRE complex, such competition would be rather infrequent under normal conditions (<10 % of elongation cycles), since the concentration of EF4 in bacterial cytoplasm is 50-fold less than EF-G (assuming that the concentration of EF-G is equal to that of the ribosome) [79,90].

EF4 associates with the cell membrane

EF4 was initially identified as a membrane protein [67]. However, since the *lepA* gene contains no membrane targeting information, the designation of LepA as a membrane protein is a matter of debate. Data from various groups have shown that EF4 associates with the membrane under non-stress conditions [42,89,91]. On the basis of the characterization of EF4-CTD [49,62] (Figure 2C), we hypothesize that this domain should interact favourably with negatively charged phospholipids, i.e. the inner membrane leaflet, via non-specific electrostatic interactions owing to the strong positive electrostatic potential [92,93]. Hence we suggest that EF4 is a membrane-associated protein under non-stress conditions rather than a membrane protein.

Another clue suggesting that EF4 is membrane-associated comes from an interaction network analysis of the whole proteome of *E. coli* [94]. EF4 was found to be involved in two classes of protein networks, namely ribosomal proteins and membrane-associated proteins (Table 1). In addition to its functions on the ribosome, EF4 might play a role in some membrane transporters, e.g. macA, yidX and jcjJ, or in integral membrane proteins, e.g. hyfE. Thus, EF4 associates with the cell membrane under optimal conditions, either by non-specific electrostatic interaction or by interaction with channel proteins/complexes.

Stress activates EF4

When *E. coli* cells encounter high ionic conditions or temperature stress, EF4 is mainly found in the cytoplasm. This indicates that the membrane is a storage location for EF4, from which it can be released as soon as it is needed by cytoplasmic ribosomes [42,88,89]. Even if the regulatory mechanism of its dissociation from the cell membrane remains unknown, we suspect that a high concentration of a cation could compete with membrane-associated EF4, thus releasing it into cytoplasm. Under optimal buffer conditions [95], EF4 has little effect on poly(U)-dependent poly(phenylalanine) synthesis [89]. When under cation or temperature stress, i.e. the concentration of magnesium is raised from 4.5 to 14 mM, EF4 stimulates poly(phenylalanine) synthesis up to 5-fold compared with translation under normal conditions, or by approximately 150 % at low temperature (20 °C) [89]. Similarly, a low molar ratio of EF4 to ribosome (1:5) can increase the fraction of active green fluorescent protein synthesized at a 12 mM magnesium concentration *in vitro* [43]. Increasing the concentration of magnesium has no significant effect on either EF-G-catalysed translocation, or on EF4 competition with EF-G for binding the PRE complex, and only has a minor effect on EF4-catalysed transfer of the POST complex to the I3 complex [77,80]. Since EF4 slows the conversion of I3 to the PRE (L) complex, this suggests that high concentrations of magnesium stabilize the interaction of EF4 with the I3 complex [80].

Magnesium plays important biological roles in the conformation and kinetic folding of RNA and/or DNA. It has the highest charge density of all biologically available ions and is the most abundant intracellular multivalent cation [96,97]. It has long been known that the structure and function of the ribosome are influenced by the presence of magnesium [98]. High concentrations of magnesium (10–15 mM) stabilize ribosome-bound tRNAs in the classical state [99], possibly impairing EF-G-dependent translocation and resulting in ribosome stalling and blocking of the upstream ribosomes on the same mRNA

Table 1 Protein interaction network of EF4 in *E. coli*

MALDI–TOF, matrix-assisted laser desorption ionization–time-of-flight; Y, yes.

Bait	Prey (locus name)	Protein name	Function	MALDI–TOF	LC-MS/MS
LepA	rplD	L4	Ribosomal protein		Y
LepA	rplI	L9	Ribosomal protein		Y
LepA	rplM	L13	Ribosomal protein		Y
LepA	rplV	L22	Ribosomal protein		Y
LepA	rplX	L24	Ribosomal protein		Y
LepA	rpsB	S2	Ribosomal protein		Y
LepA	rpsE	S5	Ribosomal protein		Y
LepA	rpsF	S6	Ribosomal protein		Y
LepA	rpsG	S7	Ribosomal protein		Y
LepA	rpsJ	S10	Ribosomal protein		Y
LepA	b1342	Hypothetical protein ydaN			Y
LepA	yeiN	Hypothetical protein yeiN			Y
LepA	yidX	Hypothetical protein yidX	Transmembrane		Y
LepA	jcjJ	Hypothetical transport protein jcjJ	Probable amino-acid or metabolite transport protein		Y
LepA	hyfE	Hydrogenase-4 component E	Integral membrane protein		Y
LepA	argA	Amino-acid acetyltransferase (EC 2.3.1.1) (N-acetylglutamate synthase) (AGS) (NAGS)			Y
LepA	b0878	Macrolide-specific efflux protein macA precursor	Efflux transporter for macrolide antibiotics		Y
LepA	recE	Restriction alleviation and modification enhancement protein			Y
LepA	LepA	LepA		Y	Y
B1248	LepA	LepA			Y

[89]. Under magnesium stress, EF4 may be released from the cell membrane via electrostatic competition, and free EF4 proteins in cytoplasm can recognize stalled POST complexes and trigger back-translocation, thereby providing a second opportunity for translocation [42,43,89]. Such a back-movement of the mRNA•2tRNAs molecule inside the ribosome is of great importance for translation fidelity and quality control of the nascent polypeptide. On one hand, such a rearrangement allows a second forward translocation that could adjust the conformation of both the mRNA•2tRNAs and the ribosome. Thus the machinery could confirm the correct reading frame on the A-site mRNA. On the other hand, such forward–backward–forward (sawing-like) action of tRNAs makes the nascent polypeptide stay longer in the exit tunnel, thus facilitating the co-translational folding of the protein product (Figure 1).

Both high magnesium [89,98] and low temperature stresses [89] can slow translation rate with ribosome stalling. Recent reports on translation factor EF-P suggest that this factor could alleviate the proline jam at the entrance of exit tunnel [100,101] by interacting with the 3′ region of P-tRNA [102]. Since EF4 interacts with the 3′ region of A-tRNA, we speculate that EF4 might thus alleviate the stalled situation of the PRE-stalling ribosomes (Figure 1). Thus, either by triggering back-translocation or by alleviating PRE-stalling, EF4 could fine-tune gene expression for an appropriate stress response.

EF4 exhibits PPIase (peptidylprolyl *cis–trans* isomerase) and chaperone activities

Our recent studies revealed that EF4 has novel enzymatic characteristics *in vitro*, possessing: (i) PPIase [55]; and (ii) chaperone activities [54]. First, the PPIase EF4 can generally catalyse *cis–trans* isomerization of proline residues on many proteins. *In situ*, EF4 catalyses specifically the isomerization of Pro^{22} from the N-terminal domain of ribosomal protein L11. In turn, Pro^{22} functions as a proline switch that regulates the recruitment and binding of EF4 to the ribosome, and eventually facilitates efficient translation [55]. Secondly, the molecular chaperone EF4 possesses general folding helper activities, such as accelerating denatured proteins refolding and postponing thermo-induced aggregation. *In situ*, chaperone activity of EF4 is especially important for maintaining the correct folding of ribosomal protein L12, and it protects the interaction between L12 and L11 to stimulate the function of EF4 on the ribosome [54].

When investigating more widely, we found that all trGTPases possess PPIase [55] and chaperone activities [54]. In addition, all conserved GTPases, no matter whether they are associated with the ribosome or not, are recognized as molecular chaperones [103]. Such enzyme characteristics of highly conserved GTPases reflect an ancient function of this class of proteins, which emerged during the early stages of evolution. Prior to the advent of proteins specifically functioning as molecular chaperones, these proteins that are often found in the close vicinity of the ribosome might be required in facilitating protein folding, especially for the newly synthesized polypeptides.

PHYSIOLOGICAL SIGNIFICANCE OF EF4 *IN VIVO*

EF4 in bacteria

Despite the high conservation of EF4 in nearly all known genomes, its biological role *in vivo* has not yet been elucidated [104]. The deletion of the EF4 gene (ΔEF4) in *E. coli* has no apparent effect on cell growth or protein export when cells are grown in nutrient-rich medium at a physiological temperature of 37 °C [81,105]. ΔEF4 cells only exhibit defective growth under pressure from the oxidant potassium tellurite and penicillin G, but have no appreciable effect on growth under other oxidizing agents [81]. ΔEF4 *Streptomyces coelicolor* cells produce more calcium-dependent antibiotics than wild-type cells, linking EF4 to antibiotic production [106]. A systematic knockout analysis carried out in *Helicobacter pylori* showed that EF4 is one of ten genes that are essential for cell survival at a hostile low pH close to the environment of stomach [107]. We therefore hypothesize

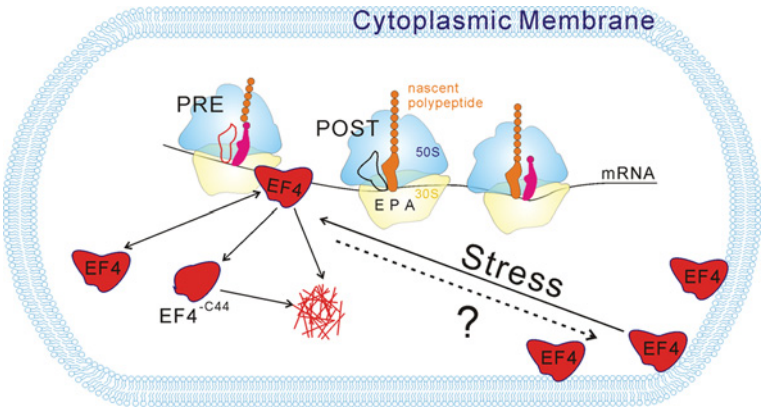

Figure 3 Models of the function and lifespan of EF4 in *E. coli* cells

EF4 produces PRE complexes either by catalysing back-translocation of the POST complex or by interacting the PRE-stalling complex. To play this role, it needs to be released from the cytoplasmic membrane reservoir (light blue bilayer molecule). This action is regulated by physiological stresses, such as high ionic conditions or abnormal temperatures. After performing its function, EF4 may re-bind to the membrane or undergo proteolysis. EF4^{-C44}, EF4 lacking the last 44 residues; empty tRNA, deacylated tRNA; orange tRNA with beads, peptidyl-tRNA; pink tRNA with one bead, amino acyl-tRNA; PRE, peptidyl and deacylated tRNA in A and P site. POST, peptidyl and deacylated tRNA in P and E site.

that, under special ionic stress, e.g. extreme pH environments for bacteria or proton gradient around a mitochondrion, the translation process is much more dependent on EF4. It also sheds light on the high conservation of the protein in mitochondria, the organelle that uses a chemiosmotic proton circuit to produce ATP.

Competition assays with ΔEF4 cells and wild-type *E. coli* cells under high Mg^{2+}, low pH or low temperature show that ΔEF4 cells are outgrown by wild-type cells within a short period [42,89]. As mentioned above, EF4 is released from the membrane upon stress (high Mg^{2+}, low temperature or high ionic strength) and it undergoes a rapid kinetic association–dissociation cycle as it performs its function in the translation machinery. As the stress subsides, EF4 may not be required any longer, and it returns to its default membrane-bound state or otherwise undergoes proteolysis [88,89]. Partial proteolysis yields a 555-residue-long intermediate in which the last 44 residues of the CTD are deleted [48] and the protein will undergo further degradation, thus bringing the life cycle of EF4 to completion (Figure 3).

EF4 in yeast

The homologue of EF4 in eukaryotes was named Guf1 (GTPase of unknown function 1) [108], and it is a conserved mitochondrial protein in all known eukaryotic genomes [43,49]. It interacts with the translating mitochondrial ribosomes [91,108,109], and appears to be important for mitochondrial protein synthesis under suboptimal conditions [91]. It is not essential and null mutants grow even better than wild-type yeast at 14°C [108]. Both overexpression and knockout of Guf1 increase the sensitivity of yeast to rapamycin [110,111]. Rapamycin is the inhibitor of TOR (target of rapamycin), which is a serine/threonine protein kinase and regulates protein synthesis and transcription to influence cell growth, cell proliferation, cell motility and cell survival [112–116]. TOR plays a crucial role for human health [117–121]. TOR integrates four major signal inputs: nutrients, growth factors, energy and stress, and transduces these signals to translation machinery by regulating 4E-BP1, the eIF4E (eukaryotic initiation factor 4E)-binding protein, and S6K1 (S6 kinase 1), the activator of ribosomal protein S6 [114]. Via phosphorylation of 4E-BP1 and/or S6K1, TOR can stimulate the initiation of protein synthesis and thus links nutrients and energy to translation. Without Guf1, the cell would exhibit severe mitochondrial defects, and therefore face a reduced energy supply for the protein translation process. This in turn would lead to suboptimal levels of protein synthesis, one of the most material- and energy-consuming reactions *in vivo* [122], and render cells sensitive to rapamycin. This possible participation of Guf1 in the TOR signalling pathway suggests that it plays a significant role in the proper control of protein synthesis and/or protein stability, particularly under nutrient/energy/redox stress conditions [54,103,123–128].

FUTURE PERSPECTIVES

Over 30 years of tremendous research efforts on the molecular function of EF4 have revealed its crucial role in the protein translation process. However, many aspects of its precise cellular function are yet to be elucidated by way of biochemical characterization *in vitro* and physiological characterization *in vivo*. Key questions still to be answered are: does it have a biological function at the the cell membrane? What regulates its release from the membrane into the cytoplasm? What are the structural and functional characteristics of its full-length CTD? For which molecular reaction is EF4 essential? What molecular roles does it play in cells of higher eukaryotes? *In vivo* studies in model organisms such as worms, *Drosophila* and mice will yield in-depth information about the precise function of EF4 in these species. Importantly, these studies will help to solve a long-standing paradox in the field of EF4 study, namely its high degree of conservation across the phyla on the one hand, and its apparent lack of phenotype in deletion mutants on the other, and should shed light on the irrefutable fact that Nature never gives up on EF4.

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