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Translation-like proteins in bacterial dormancy and ribosome hibernation

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Abstract

Translation factors are among the most conserved proteins in nature. Yet nearly half of studied bacterial species also carry catalytically dead paralogs of two such factors, elongation factor EF-G and release factor aeRF1. These paralogs, known as EF-G2 and Balon, have a debated biological activity. Here, we summarise emerging evidence that they help bacteria store inactive ribosomes during hibernation, enabling them to survive starvation and other hostile conditions.

Protein EF-G: a one trick pony?

In his book, *The Conduct of Inquiry*, published in 1964, the philosopher and behavioral scientist Abraham Kaplan warned the scientific community against overreliance on a single method of research: when we look at something from a single point of view, we inevitably see it in a one-sided and, at times, deceiving way[1]. Two years later, Abraham Maslow, the author of the popular pyramid of human needs, polished Kaplan's ideas to coin a now-famous proverb: "if all you have is a hammer, everything looks like a nail."[2] Therefore, in studying any subject, it is not enough to know the current knowledge; we must also understand how this knowledge was produced, and by whom. Such history allows us to inherit the wisdom of the past without inheriting its biases and blind spots, and to approach the unknown with open eyes and an open mind, ready to discover what we do not even know we do not know.

The same is true about the universally conserved protein known as elongation factor EF-G. Currently, every student of molecular biology can tell the textbook definition that EF-G is a conserved protein that stimulates protein synthesis by helping ribosomes move along mRNA[3]. However, not so many, even among the leaders of the field, know how EF-G was discovered, and how the history of its discovery shaped the way we study and think about this ubiquitous protein today.

EF-G was discovered at the corner of 66th Street and York Avenue, Manhattan, in a series of works published between 1962 and 1969, by Fritz Lipmann and his colleagues, in the famous Flexner Hall of the Rockefeller University[4]. Lipmann joined the Rockefeller in 1957 as an established titan of molecular biology who over his entire career was obsessed with figuring out how living cells use and store chemical energy. His work, recognized by the 1953 Nobel Prize in Physiology or Medicine, revealed, among other things, that living cells use molecules like ATP to store energy and drive many chemical reactions that sustain life[5]. When Lipmann moved his laboratory from Massachusetts General Hospital to Rockefeller, he found himself neighboring the lab of George Palade, who had discovered ribosomes just two years earlier[6]. Influenced by this proximity, Lipmann changed the focus of his work to investigate how the energy in the cell is used by ribosomes to produce proteins.

Over the following decade, Lipmann's lab showed that ribosomal protein synthesis requires hydrolysis of GTP molecules, and that the amount of GTP hydrolyzed is strictly proportional to the amount of protein produced by ribosomes in cell lysates[7,8]. Furthermore, they then determined that the GTP-hydrolyzing activity is not a feature of the ribosome itself, but is carried out by at least one cellular protein that binds

the ribosome during protein synthesis[8,9]. They named this protein factor G, for GTPase, which was later renamed elongation factor G (EF-G)[8,10].

This finding laid the rails along which EF-G research would run for decades. In 1969, Spirin proposed the ribosome ratchet model, in which EF-G was suggested to use the irreversible hydrolysis of GTP to drive the unidirectional movement of the ribosome along the mRNA, advancing one codon per GTP molecule[11]. This model was later validated, refined and elaborated by an avalanche of biochemical, mutational and structural studies, which revealed how EF-G recognizes the ribosome[12], how EF-G binds GTP and catalyzes its hydrolysis[13,14], and how EF-G activity can be targeted by antibiotics, such as fusidic acid or argyrin B, to arrest ribosomes in the middle of protein synthesis[12,15,16]. It was also established that, after protein synthesis terminates, the GTPase activity of EF-G helps to stimulate the last step of protein synthesis: splitting ribosomes into subunits so they can reinitiate protein synthesis[17,18]. Furthermore, it was shown that EF-G is conserved in every studied organism, and its homologs in archaea and eukaryotes (aEF2 and eEF2, respectively) perform essentially the same catalytic role in the process of protein synthesis[19].

Thus, for over half a century, EF-G has been studied almost exclusively through the lens of protein synthesis. To paraphrase Maslow: if all you study is translation, every function of EF-G looks like a translation factor. Yet is translation really all EF-G does?

A wicked twin: EF-G has a catalytically null paralog, EF-G2

By the late 2000s, analyses of the growing number of sequenced genomes had revealed that nearly half of bacterial species carry not one but two copies of the EF-G gene. In some species, primarily in δ-Proteobacteria (e.g. *Desulfotalea psychrophila*) and Spirochaetes (e.g. *Borreliella burgdorferi*), both copies, which likely emerged via evolutionarily recent gene duplications, appeared to encode catalytically active variants of EF-G, as suggested by the high conservation of all structural elements required for GTP binding and hydrolysis. However, in the overwhelming majority of the species bearing two EF-G paralogs, only one gene, namely *fusA*, appeared to encode a catalytically active isoform (EF-G1), whereas the second gene, namely *fusA2*, encoded a protein (EF-G2) lacking the catalytic residues or even entire protein segments that correspond to the catalytic switches I and II[20,21]. Direct biochemical examination, initially performed for EF-G2 from *Mycobacterium smegmatis*, revealed that, consistent with its structural features, EF-G2 indeed lacked the ability to hydrolyze GTP in a ribosome-dependent manner[22], pointing to the widespread existence of catalytically null paralogs of EF-G in bacterial cells.

Phylogenetic analyses of EF-G paralogs revealed that EF-G2 most likely emerged via an ancient event of EF-G gene duplication in the common ancestor of modern bacteria, but was later lost in some lineages (e.g. most Bacteroidetes) and retained by ~40% of the representative bacteria (**Fig. 1**). Furthermore, our analysis performed for this commentary showed that this isoform, EF-G2, has a strictly conserved domain composition: in all representative bacteria, EF-G2 contains each of the five domains present in

EF-G1. We also found that — although EF-G2 lacks the domain I residues required for GTP hydrolysis and association with the GTPase-associated center of the ribosome — it retains high conservation of residues in domain IV (specifically, residues 507–513 of loop I and residues 583–589 of loop II, including His583, Ser588 and Glu589, in *E. coli* numbering) that allow EF-G1 to recognize the anticodon of the peptidyl-tRNA in the P site during ribosome translocation (**Fig. 2**). Thus, EF-G2 turned out to structurally resemble EF-G1 and retain the conserved residues required for association with the mRNA channel and the ribosomal P site, yet lack the residues involved in GTP binding and association with the GTPase-associated center of the ribosome.

These findings presented a paradox: why would over 40% of studied bacteria retain a “molecular zombie” of EF-G throughout what is estimated to be ~3.9 billion years (!) of bacterial evolution?

The most common activity is no activity at all: EF-G2 as a hibernation factor?

While many labs around the world were exploring how ribosomal protein synthesis is stimulated by EF-G, another line of research has quietly emerged to reveal an unexpected, yet the most common and completely neglected activity of the ribosome, which turned out to be no activity at all[23]. First, global studies of microbial metabolism revealed that even in relatively nutrient-rich natural environments, such as soil, over 90% of bacterial cells exist in various forms of dormancy[24,25]. The proportion of dormant microorganisms was estimated to be even higher in growth-limiting environments, including permafrost, the deep subseafloor and deserts[26–29]. It was inferred from these studies that the overwhelming majority of microbial biomass on our planet exists in various forms of dormancy, meaning that most bacterial cells at a given moment in time do not use their ribosomes for protein synthesis but store them in an inactive form.

What’s more, studies of ribosomes isolated from dormant bacteria revealed that inactive ribosomes associate with an evolutionarily diverse array of protective proteins, now known as ribosome hibernation factors, that help bacterial cells to safely store ribosomes until conditions improve[30–34]. Some of these proteins, such as the *E. coli* protein RaiA, were found to be highly abundant even in actively growing cells (~7,900 copies per cell) and activated as cells experience stress or transition to dormancy. Other hibernation factors, such as *E. coli* RMF, were found to accumulate gradually as cells transition from active growth to stationary phase (from ~200 to ~3,500 copies per cell)[35].

Importantly, ribosome hibernation factors were shown to increase the metabolic stability of ribosomes in dormant bacteria. When hibernation factors bind vacant ribosomes, they typically stimulate the association of the individual subunits of inactive ribosomes into either hibernating 70S ribosomes or hibernating ribosome oligomers (dimers, tetramers, ribosome sheets or even ribosome microcrystals), thereby shielding a substantial part of the functional ribosome surface from nucleolytic attack[34,36]. This protection was shown to save bacterial cells the colossal amount of energy required for the synthesis of new ribosomes and to give them the competitive advantage of being able to rapidly restore a high rate of

protein synthesis, and hence rapid bacterial replication, when conditions improve and starved bacteria resume their competition for nutrients[33,36].

What's more, studies of eukaryotic ribosomes revealed that hibernating ribosomes in various species, including humans, associate not only with hibernation factors but also with translation factors, including the eukaryotic homolog of EF-G, eEF2[37,38]. One of the most striking observations was that eEF2 is often more abundant on dormant than on translating ribosomes, where it associates with the hibernation factor SERBP1 in nearly stoichiometric amounts[38,39]. These findings, along with the nearly stoichiometric association of eEF2 with hibernating ribosomes, suggest that the most commonly occurring biological activity of eEF2 may be not to promote protein synthesis through translocation but to help cells store their inactive ribosomes and possibly recover them when conditions improve. In other words, had eEF2 been discovered by researchers studying ribosome hibernation, it would most likely have been named a factor of ribosome storage or dormancy, not an elongation factor.

Studies of gene expression in mycobacteria revealed that the same may be true for EF-G2: this catalytically null protein may function as a ribosome hibernation factor rather than a translation factor[22,40]. Specifically, its expression was found to be activated in the late stationary phase of *Mycobacterium smegmatis*, and during carbon starvation in *Bacteroides thetaiotaomicron*[22,41,42]. Furthermore, genetic knockouts in *M. smegmatis* revealed that strains lacking EF-G2 were gradually outcompeted by wild-type strains in growth competition assays during stationary phase[22]. Finally, isolation of ribosomes from stationary-phase cells revealed that EF-G2 associates with a fraction of 70S ribosomes and also binds to individual 50S subunits (two copies of EF-G2 per 50S subunit) — a phenomenon of unclear physiological relevance, yet one showing a previously unappreciated ability of EF-G2 to engage with inactive ribosomal particles[40].

Thus, eEF2 and EF-G2 were both shown to respond to growth-limiting conditions by forming complexes with ribosomes that are consistent with these proteins serving as ribosome hibernation factors — the idea completely unforeseen during the early studies of EF-G, the idea that may explain the high prevalence of EF-G2 across bacteria, and the idea that has been commonly neglected in the past and present research due to the dominant bias to see EF-G-type proteins first and foremost as translation factors.

EF-G2 is not alone: some bacteria possess other translation-like factors involved in ribosome hibernation

In 1967, shortly after Lipmann's discovery of EF-G at the Rockefeller, Mario Capecchi, then a graduate student in James Watson's laboratory at Harvard, identified another essential factor of protein synthesis: the release factor[43]. This protein was first found in bacteria, where it was characterized as two paralogs, RF1 and RF2, encoded by two different genes and recognizing different sets of stop codons[44]. A few decades later, an analogous protein, a/eRF1, was identified in archaea and eukaryotes and proved

structurally unrelated to the bacterial release factors, yet similar to them in overall shape and mechanism of action[45,46].

In archaea and eukaryotes, proteins of the a/eRF1 family were found to carry two highly conserved motifs, NIKS and GGQ[47,48]. Structural studies revealed that the NIKS motif allows a/eRF1 to bind the ribosome when a stop codon occupies the A site, because it can recognize all three stop codons in archaeal and eukaryotic mRNAs[46,49]. The second motif, GGQ, was found to reach the ribosome's catalytic center, the peptidyl transferase center[46,49]. There, its glutamine positions a water molecule to hydrolyze the ester bond between the nascent peptide and the P-site tRNA, which releases the newly synthesized protein[47,50].

Then, as with EF-G2, comparative genomics studies revealed that many bacterial species carry homologs of a/eRF1[51,52]. These proteins were collectively named the baeRF1 family[51,52]. However, sequence analysis revealed two key differences between the bacterial baeRF1 proteins and their archaeo-eukaryotic counterparts: the bacterial proteins turned out to lack both the NIKS and GGQ motifs invariably found in a/eRF1, due either to amino acid substitutions or to complete deletions of the corresponding protein segments[52,53]. Furthermore, analysis of their genomic context revealed that baeRF1 genes in bacteria are often located next to genes encoding hibernation factors, suggesting a role in ribosome hibernation[52,53]. This role was corroborated via proteomic studies of ribosomes from *Mycobacterium tuberculosis*[54] and directly confirmed by cryo-EM of ribosomes from *Psychrobacter urativorans*[53] and *Synechocystis sp. PCC 6803*[55], and the protein was renamed Balon.

The structural studies showed that Balon binds the ribosomal A site in a manner consistent with a role as a hibernation factor. In one complex, isolated from stationary-phase or cold-shocked bacteria, Balon was found bound to vacant ribosomes together with the hibernation factor RaiA[53]. In another, Balon occupied the A site of ribosomes that still carried mRNA and peptidyl-tRNA: due to the absence of the NIKS motif, Balon did not need to read a stop codon and could bind ribosomes regardless of whether mRNA was present. Furthermore, in the absence of the GGQ motif, Balon could not trigger peptide release, so the nascent peptide stayed attached to the P-site tRNA[53]. Together, these findings suggested that Balon can initiate hibernation of ribosomes stalled on mRNAs, preserving them in a translation-competent state during stress.

Thus, bacteria turned out to possess at least two proteins, EF-G2 and baeRF1/Balon, that resemble translation factors yet lack their catalytic residues. Both were found to retain the overall structural scaffold of their ancestral factors, along with the residues needed to bind the ribosome. This enables them to associate with dormant ribosomes and likely act as hibernation factors, protecting ribosomes from degradation while cells are not using them for protein synthesis.

What's next? How many translation-like factors exist in nature disguised as translation factors, and what is their full spectrum of

biological activities?

It is obvious that this commentary was written by scientists who see the world through the prism of dormancy, and who are therefore prone to the very bias we described above: to see the role of translation-like factors primarily as ribosome hibernation factors. Therefore, it is important to ask: what is the full spectrum of biological activities carried out by these ancient proteins?

We do not yet know the extent of the unknown in biology and nature, and one thing we should stop doing is explaining new evidence with old models, especially when the data do not fit them. It is like listening to someone read a complex novel aloud and trying to write down what we hear using only the three letters of the alphabet we have learned so far: however cleverly we combine As, Bs and Cs, we will capture only tiny fragments of the story, misinterpret much of it, and may come to believe that these fragments are the novel itself. Proteins such as EF-G2 and Balon may be the first signs of letters we have not yet learned. Rather than spelling them with the As, Bs and Cs we already know, we should stay attentive, and excited, whenever new evidence suggests that the story is bigger than our alphabet.

Declarations

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Author contributions

A.v.d.E. and S.V.M. revised the current data on EF-G2 and Balon/baeRF1 conservation and wrote the manuscript.

Competing interests

The authors declare no competing interests.

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Figures



Figure 1

EF-G2 and Balon are widespread and understudied bacterial proteins. The phylogenetic tree and plot show that ~40% of the analyzed bacterial species encode two variants of EF-G: EF-G1, a well-characterized and catalytically active translation factor required for protein synthesis, and EF-G2, a widespread but poorly characterized paralogue of EF-G1 that is predicted to be catalytically inactive and is likely involved in ribosome hibernation. Additionally, many bacterial species encode a homologue of the archaeo-eukaryotic release factor aeRF1, known as baeRF1 or Balon. Circle size indicates the fraction of species in each lineage that carry each gene; dot plots show copy number among carriers. Tree topology is taken from ref. 56; clade widths are not to scale for simplicity.

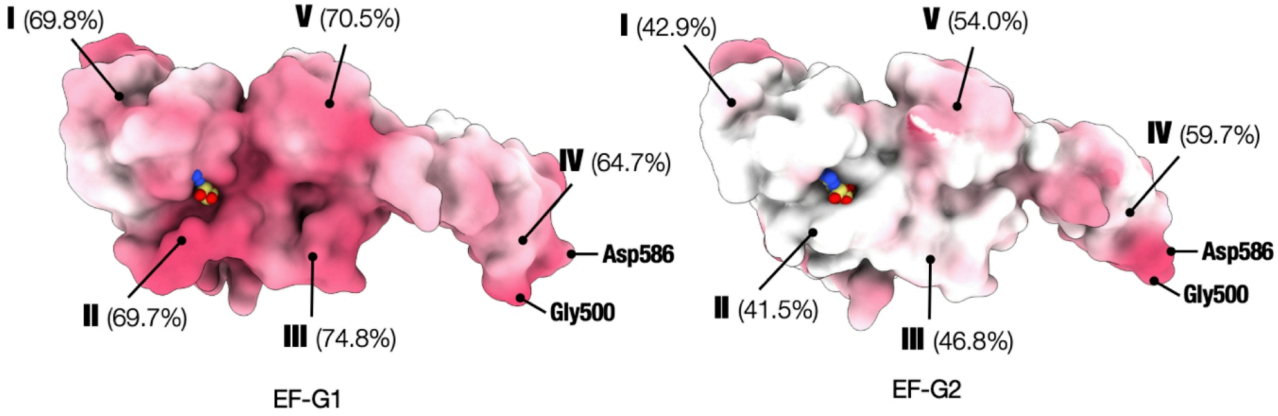

Figure 2

EF-G2 is a catalytically null variant of EF-G, possibly acting as a ribosome hibernation factor. Structures of EF-G1 from *Mycoplasma pneumoniae*(PDB 7PAQ) and EF-G2 from *Mycobacterium smegmatis* (PDB 9K0Z), colored by sequence conservation across 814 bacterial species that encode both proteins (with white corresponding to poorly conserved segments (<30% average pairwise sequence identity) and pink corresponding to highly conserved segments (≥80% average pairwise sequence identity)). The aligned structures are shown as seen by the 50S ribosomal subunit, both showing a GDP molecule to indicate the position of the nucleotide-binding pocket (as spheres). The actual GDP molecule is present only in the structure of EF-G1. The figure illustrates that both proteins have conserved sites required for binding to the P-site tRNA (domain IV loops 507–513 and 580–587, *Escherichia coli* numbering, identical in EF-G1 and EF-G2). EF-G1 also shows strict conservation of the sequence motifs required for GTP binding and hydrolysis during protein synthesis. By contrast, EF-G2 has a poorly conserved sequence of protein segments required for the catalytic activity in EF-G1. These conservation patterns are consistent with experimental findings in *M. smegmatis* showing that, unlike EF-G1, EF-G2 lacks ribosome-dependent GTPase activity and cannot stimulate ribosome recycling. Overall, this figure illustrates that many bacterial species bear two homologs of EF-G: catalytically active EF-G1 for protein synthesis, and catalytically null EF-G2 possibly mediating ribosome hibernation.