Ribosome pausing, arrest and rescue in bacteria and eukaryotes

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Ribosomes translate genetic information into polypeptides in several basic steps: initiation, elongation, termination and recycling. When ribosomes are arrested during elongation or termination, the cell’s capacity for protein synthesis is reduced. There are numerous quality control systems in place to distinguish between paused ribosomes that need some extra input to proceed and terminally stalled ribosomes that need to be rescued. Here, we discuss similarities and differences in the systems for resolution of pauses and rescue of arrested ribosomes in bacteria and eukaryotes, and how ribosome profiling has transformed our ability to decipher these molecular events.

This article is part of the themed issue ‘Perspectives on the ribosome’.

1. Introduction

Although the core mechanisms for the translation of mRNAs into their encoded protein products are conserved, cells have different strategies for regulating translation and imposing quality control. In bacteria, ribosomes engage mRNAs co-transcriptionally as there is no partitioning of the cell to segregate transcription and translation. Ribosomes can identify start sites in an mRNA as soon as the transcript exits RNA polymerase and thus begin translation independent of any quality control steps to ensure that the mRNA is complete. In eukaryotes, mRNAs are processed in the nucleus prior to export to the cytoplasm. As a result of this separation and a distinct mode of translation initiation, eukaryotes leverage several types of quality control before the ribosome even begins translation. More specifically, mRNA capping and polyadenylation (and splicing in higher eukaryotes) are critical for both export from the nucleus and efficient translation initiation. Through these early quality control steps, the eukaryotic translational machinery is strongly biased to engage mRNAs that are complete. Thus, these fundamental differences in cell structure and in the mechanism of translation initiation for bacteria and eukaryotes lead to very different extents of quality control at the initiation step of translation.

Once initiation is complete, however, the challenges are more similar in both systems—the ribosome must successfully elongate through the open reading frame (ORF) until a stop codon is encountered. In bacteria, where initiation takes place independent of evaluation of downstream mRNA integrity, it is relatively common for the ribosome to run to the end of a truncated transcript that has been generated by mRNA cleavage [1]. In eukaryotes, the presence of an intact cap and poly-A tail strongly stimulates initiation, guarding against this problem to some extent, but mRNA cleavage generates truncated mRNA templates in eukaryotes also. Truncated mRNAs cause obvious problems: the protein product is incomplete and may have dominant negative or toxic activities, the mRNA is not worth translating a second time, and ribosomes are sequestered in the absence of the standard mechanism of recycling at stop codons. Such crises are resolved both in bacteria and in eukaryotes. But, just as their canonical termination and recycling mechanisms are quite distinct from one another, so too are the bacterial and eukaryotic solutions for resolving stalled ribosome complexes.

Ribosomal arrest on truncated messages is not the only threat to translation: more subtle perturbations in elongation are also detected in both the bacterial and eukaryotic systems, though it has been challenging to differentiate between...
what constitutes a major crisis in which the mRNA and nascent peptide need to be targeted for destruction, and what constitutes a minor crisis that can be resolved more productively. Again, the systems in bacteria and eukaryotes for implementing these distinct choices have some commonalities but many more differences.

Here, we will compare and contrast the quality control processes that monitor translation in bacteria and eukaryotes. There has been tremendous progress in recent years in understanding the molecular basis for these events, though much still remains to be deciphered. Ribosome profiling has added a critical tool for the evaluation of these pathways in both systems, though there remain technical challenges that still limit the resolution of deep sequencing approaches. We will discuss these successes and challenges.

2. Comparisons of translation in bacteria and eukaryotes

Despite broad conservation of the translational machinery (the ribosome, tRNAs and core protein factors) from bacteria to man, the molecular specifics of some steps are more conserved than others. Translation initiation is quite distinct in bacteria and eukaryotes. In bacteria, ribosomes find the start codon (AUG or GUG) either based on proximity to a Shine-Dalgarno (SD) motif or using a leaderless initiation process where the AUG is found very near to the 5' end of the mRNA [2]. By contrast, eukaryotic ribosomes engage with mRNAs possessing a cap and a poly-A tail, and then ‘scan’ for AUG starting from the 5' end [3]. As noted above, the ramifications of these differences for overall gene expression and quality control are substantial.

Elongation is the most similar step in translation. The core processes of tRNA selection, peptide bond formation and translocation are catalysed by homologous factors—EFTu/eEF1A, the large subunit peptidyl transferase centre and EFG/eEF2—acting in a homologous fashion. Recent studies from a number of groups have identified another common elongation factor—EFP/eIF5A—that at a minimum promotes peptide-bond formation on problematic iterated proline stretches in both systems [4–6]. Finally, there is an essential fungal-specific elongation factor, eEF3, an ATPase that is proposed to facilitate departure of the E-site tRNA during each round of elongation [7]. There has been some speculation that more distantly related ATPases could play a similar role in higher eukaryotes or even in bacteria. Broadly speaking, however, the process of translation elongation is quite conserved between the kingdoms of life, consistent with a common origin.

Finally, translation termination and recycling are not well conserved between bacteria and eukaryotes (figure 1). Both systems encode ‘STOP’ with three codons—UAA, UAG and UGA—but the factors that recognize these codons are unrelated [8]. In bacteria, tRNA-like termination factors RF1 and RF2 recognize an overlapping set of stop codons (UAA/UAG and UAA/UGA, respectively) with high specificity. RF3, a GTPase related to EFG, is found in some bacteria and appears to facilitate the departure of the E-site tRNA during each round of elongation [9,10]. By contrast, in eukaryotes, a single termination factor called eRF1 recognizes all three stop codons. Again, a GTPase is involved; eRF3 is a GTPase, in this case related to EFTu, that facilitates loading of eRF1 into the A site for catalysis [11,12].
Importantly, eRF1 is not homologous to RF1/RF2, and eRF3, while a GTPase, is only distantly related to RF3. These data are most easily reconciled by arguing that termination evolved independently after the divergence of these major lineages.

Ribosome recycling in bacteria and eukaryotes is wholly distinct (figure 1). Recycling in bacteria depends on a specialized ribosome recycling factor (RRF) that works together with EFG to promote subunit dissociation after termination factors have departed [13]. In eukaryotes, recycling is promoted by eRF1, which remains bound following peptide release, working with the ABC-family ATPase Rli1/ABCE1 [14–16]. Thus, these processes differ both in the specific factors utilized and in the extent of coupling between the termination and recycling events. As we shall see, these differences impact how quality control systems in both lineages identify defective translation elongation and termination events and target them for processing.

3. Ribosome profiling: breakthroughs and challenges

Ribosome profiling is a genome-wide method for defining ribosome occupancy on mRNA transcripts by sequencing ribosome-protected fragments (RPFs or footprints). This approach, introduced in 2009 by Ingolia & Weissman, has revolutionized work in the translation field [17]. For the first time, researchers can observe ribosome activity in living cells with close to single-nucleotide resolution. Although the technique is often used to quantitate differences in gene expression by counting the total number of ribosomes per gene under two or more conditions, it has also proved to be very powerful in defining in vivo functions of individual components of the protein synthesis machinery itself. Ribosome profiling studies have shed light on alternative initiation sites [18,19], conformational changes in elongating ribosomes [20], frame-shifting [21], stop codon readthrough [22], tRNA modifications [23,24], ribosome-associated chaperones [25,26], and, as will be discussed below, rescue and recycling factors [27,28].

In principle, ribosome profiling has the capacity to reveal pausing sites throughout the transcriptome with unprecedented clarity, and when a kinetic layer is added, to see the actual dwell times of ribosomes as translation occurs [19]. In practice, however, individual pauses are difficult to identify given the high levels of noise arising from biases in the cloning process [29]. Peak intensities vary by as much as 1000-fold across highly translated genes. Calculation of robust pause metrics requires the averaging of many instances of a pause motif genome-wide to minimize noise (using average or meta-gene analyses). And, depending on the motif being studied, the number of instances may be insufficient to reveal such trends.

Given that the ribosome profiling method is still in its early days, aspects of the protocol that can lead to bias are still being identified and ironed out. Consider, for example, the size of the ribosome footprints in profiling experiments. In early studies, it was found that the abundant 28 mer footprints in yeast contained the most information about ribosomal position and reading frame [17]. Indeed, when analysis is limited to these fragments, more than 95% of reads map to the first position in codons, allowing for ready analysis of reading frame and other high-resolution phenomena [27]. In bacteria, however, a far broader distribution of fragment lengths is observed, ranging from 15 to 40 nts in length. By sampling only the upper part of this distribution, footprints 28–42 nt in length, one of the earliest bacterial ribosome profiling studies inadvertently enriched for RNA fragments containing SD motifs, which tend to be longer than those without SD motifs [30]. As a result of this artificial enrichment, ribosomes appeared to pause within ORFs at SD motifs. It turns out that these pauses are weaker or even absent when the full range of ribosome footprints is cloned and sequenced, suggesting that SD motifs are not a primary determinant of ribosome pausing in bacteria [31].

Another critical factor in the ribosome profiling method is how ribosomes are prevented from moving during cell lysis and subsequent mRNA digestion. In early papers, cycloheximide (in eukaryotes) or chloramphenicol (in bacteria) were added to cultures prior to lysis [17,25]. These general elongation inhibitors cause ribosomes to accumulate at start codons (and more generally the 5' end of genes) as elongation grinds slowly to a halt while initiation continues unimpeded [32]. Importantly, any translation in the extract in the presence of antibiotics (cycloheximide or chloramphenicol) leads to codon or amino acid–specific pauses related in part to these specific agents [31,33,34]. Many later studies have not included antibiotics in the media but sought to minimize distortions from the in vivo state by harvesting and flash-freezing cells rapidly. Antibiotics are only added in the lysis step to prevent potential ribosome movement at this later stage. Even so, it is clear both in yeast and in bacteria that there is some ongoing translation even in these carefully prepared samples, and that this movement can preclude the direct observation of what is happening at the exact moment the cells are harvested (AR Buskirk 2016, unpublished data).

As the protocol is improved to remove sources of bias, ribosome profiling will yield more and more accurate information about ribosomal pausing in vivo. The challenge is in establishing a correlation between the pauses observed by profiling and some agreed-upon pausing signal. For example, amino acid depletion leads to clear pauses at the corresponding codons due to the anticipated delay in recruiting aminoacyl-tRNAs that are in low concentration in the cell. These pauses are particularly clear in samples prepared without translation inhibitors [20,27]. There has been much controversy surrounding the conflicting data from ribosome profiling studies with regard to ribosome pausing at rare or non-optimal codons under normal conditions [23,35–38]. While the field anticipated that the pausing profiles would correlate with codon optimality, the data have failed in general to show a robust correlation. However, a recent meta-analysis of eukaryotic profiling data shows that, in the absence of cycloheximide in the culture media, ribosomes do discernibly pause at non-optimal codons as expected [33]. This study goes a long way to resolving this controversy, bringing the profiling data in line with previous genetic and bioinformatic observations, and gives hope that analyses of pausing and stalling will become increasingly powerful in the future.

4. Pauses that are reversible

The length of translational stalling events varies all the way from fleeting pauses that are quickly resolved to dead stops where the ribosome is irreversibly arrested (figure 2). The translational quality control machinery must differentiate between pauses that are pathological and require intervention and those that pose no threat to the cell or are even adaptive.
Pauses on stretches of codons that are decoded slowly, for example, regulate the transcription of downstream genes in certain bacterial leader peptides in biosynthetic operons [39]. In a similar manner, non-optimal codons in structural genes have been implicated in fine-tuning translational rates in order to favour correct protein folding [40–42]. Nothing prevents ribosomes from proceeding when the cognate aa-tRNAs become more available. In these examples, the mRNA sequence has evolved to encode more information than just the protein sequence, slowing the rate of translation in a programmed fashion.

The concentration of aa-tRNAs is not the only factor that affects the rate of translation; the sequence of the nascent peptide itself also plays a role. If we consider the termination reaction, for example, the final two or three amino acids can dramatically inhibit peptide release. Short motifs comprised of small, polar residues seem to effectively slow release catalysis [43]; tellingly, threonine is underrepresented at the final codon in bacterial proteomes. Hydrolysis of peptides ending in proline also appears to be problematic, especially when another proline or a negatively charged residue is positioned just upstream (i.e. proteins ending in DP or EP) [44].

The interaction of nascent peptides and the ribosome can induce pauses that serve important regulatory functions. Stalling during synthesis of the TnaC peptide, for example, regulates the transcription of downstream genes in response to changes in the concentration of tryptophan [45]. Free tryptophan binds the ribosome and inhibits release of TnaC by RF2; three conserved residues in the nascent peptide are required for this activity, including the C-terminal Pro residue. Another example is the SecM peptide, where stalling regulates the translation of downstream genes in response to changing levels of activity of the secretory machinery [46]. In this case, stalling occurs during elongation rather than termination; ribosomes pause at the RxGP motif with the Gly codon in the P site and the Pro codon in the A site [47]. Premature processing of the stalled TnaC and SecM complexes by ribosome rescue factors would interfere with these programmed regulatory mechanisms and needlessly terminate productive protein synthesis. In each of these cases, the presence of aa-tRNA or release factors in the A site precludes the quality control machinery from recognizing these complexes [47,48].

In TnaC, SecM and other known stalling peptides, proline poses a challenge to the ribosome; Pro is both a poor peptidyl donor and a poor peptidyl acceptor. The fact that proline is a secondary amine explains its slow kinetics as a peptidyl acceptor (i.e. Pro-tRNA in the A site reacting with the nascent polypeptide) [49]. The constrained geometry of the cyclic proline side chain probably explains its poor reactivity as a peptidyl donor in elongation and peptide release. These two characteristics of the chemistry of proline combine to make stretches of two or more consecutive prolines difficult for translating ribosomes. It is reasonable to think that all of these constraints are part of the equation that determines the evolution of protein sequences.

5. Factors that resolve pausing

In bacteria, the synthesis of proline-rich motifs is made possible by EFP [5,6], a factor that binds between the canonical E and P sites and contacts the peptidyl-tRNA. Simply viewed, EFP structurally mimics both the acceptor and anticodon stems of canonical tRNAs and contacts the mRNA in the E site [50]. In many bacteria, a key lysine residue is modified with a β-lysine moiety [51,52]; in others, arginine side chains are modified with other small molecules [53,54]. How do these modified side chains enhance the reactivity of peptidyl-tRNAs ending in proline? Careful kinetic analyses reveal that EFP reduces entropy in the peptidyl transfer reaction rather
than making enthalpic contributions [55]. EFP thus stabilizes an optimal geometry of the peptidyl-tRNA through its interactions with the CCA-end rather than contributing to catalysis more directly. In support of these ideas, the modified side chains in some bacterial EFP proteins are too short to reach the reactive species in the peptidyl transferase centre in the ribosome but are probably able to contact the CCA moiety of the peptidyl-tRNA [55].

In eukaryotes and archaea, an EFP homologue named e/eIF5A promotes peptidyl transfer at polyproline stretches [4]. Originally implicated in initiation based on its ability to stimulate a model reaction with puromycin and Met-tRNA [56], more recent studies have argued for a broader role in elongation [57]. eIF5A also contains a post-translationally modified residue, hypusine, that is positioned proximal to the CCA-end of the peptidyl-tRNA [58]. Lacking the domain in EFP that resembles the anticodon stem, however, eIF5A mimics only the acceptor stem of tRNA. eIF5A was found to be stably bound to translating ribosomes when cells were treated with cycloheximide, destabilizing the binding of E-site tRNA [58]. Given that both EFP and eIF5A must enter the ribosome through the E site, the dissociation of E-site tRNAs may serve as a signal of slow translation that requires intervention. Importantly, while EFP is non-essential in many bacteria, eIF5A (and the enzymes that catalyse its modification) is essential in yeast and higher eukaryotes. This requirement may be due to the fact that a far higher percentage of eukaryotic proteins have polyproline motifs (10%) or because it functions more generally.

In a ribosome profiling study of E. coli cells lacking EFP, we observed strong pauses (more than 10-fold above the average ribosome density) at roughly half of the Pro–Pro motifs [59]. These pauses were also observed in bacteria lacking EpmA and EpmB, the enzymes responsible for post-translational modification of the conserved lysine residue. Levels of pausing at Pro–Pro motifs varied 100-fold on the subsequent codon: for example, PPW, PPN, FPD and PPP induced very strong pauses while PPI, PLL, PPM, FPP and PPP exhibited no pausing. Pausing at these motifs occurs with both prolines already incorporated in the nascent peptide and the third codon in the A site. Interestingly, we found that the codon before the Pro–Pro motif also matters: GPP, DPP and APP pause particularly strongly when Gly, Asp and Ala codons are positioned in the E site.

What effect do these pauses have on protein output? In the absence of EFP, average ribosome density is reduced downstream from polyproline motifs, though curiously this effect is far stronger on some genes than on others. In general, genes that have high initiation rates and proline pauses close to the 5′-end of the gene tend to have reduced ribosome density downstream [59]. One model that could explain this phenomenon is that ribosomes form a short queue behind the stalled ribosome, sterically blocking initiation and lowering protein output [60]. Indeed, we observed one or two stacked ribosomes behind the lead ribosome stalled at the polyproline motif. These data begin to tie the biochemical activities of EFP to its broader role in gene expression in vivo.

Although EFP adequately resolves pauses at polyproline motifs, it does not suppress stalling on endogenous leader peptides that regulate gene expression in bacteria. Although stalling at both peptides depends on a carefully positioned proline residue, extensive interaction with upstream amino acid residues within the exit tunnel of the ribosome apparently provides enough energy to block peptidyl transfer (in the case of SecM) or peptide release (in the case of TnaC) even in the presence of EFP. In the absence of the right cellular conditions to relieve these stalling events, the ribosomes are permanently arrested. Indeed, SecM-stalled complexes are exceptionally stable in the absence of a signal peptide sequence that allows the secretory machinery to effectively pull the peptide out of the ribosome mechanically [61]. As discussed below, one challenge that translational quality control machinery faces is to distinguish between such productive stalled complexes and irreversibly arrested complexes.

6. Stops that trigger rescue (or destruction)

More troublesome than a slow stop during translation elongation is a dead stop (figure 2). A clear example of a dead stop occurs when ribosomes reach the 3′ end of an mRNA without encountering a stop codon. Such mRNAs are commonly produced by endo- or exonucleolytic cleavage events in the cell that accompany either normal mRNA decay or quality control processes that we describe below. Both bacteria and eukaryotes have dedicated systems for processing such defective ribosome complexes. Three basic events typically occur—the incomplete protein product and aberrant mRNA are targeted for decay and the ribosome is ‘rescued’ from this trapped state [62]. The diagnostic molecular feature for both bacteria and eukaryotes for this class of targets is an A site lacking an mRNA template, and therefore unable to engage normal substrates whose binding depends on codon recognition (aa-tRNAs, release factors). What enters this site to resolve the crisis is strikingly different in these systems, but, importantly, both systems have been shown to act optimally on truncated mRNAs.

7. Bacterial crisis rescue

In bacteria, the primary system for rescuing ribosomes stalled on truncated mRNAs comprises transfer-messenger RNA (tmRNA) and its accessory protein SmpB (figure 3) [63,64]. This system is encoded in the genomes of all free-living bacteria, suggesting that it is an ancient and effective solution to this particular problem [65]. Together, tmRNA and SmpB rescue stalled ribosomes in a process referred to as ‘trans-translation’ (for reviews, see [66,67]). tmRNA, as its name implies, functions first as an aminocacylated-tRNA, binding to the empty A site and adding an alanine to the nascent peptide chain in a standard peptidyl transferase reaction. SmpB promotes accommodation of the complex through its interaction with the tmRNA channel [68]. tmRNA then functions as an mRNA, binding within the decoding centre and directing the synthesis of a short peptide. Because tmRNA encodes a short ORF that ends at a stop codon, normal termination and ribosome recycling can occur, thus resolving the dead-end ribosome complex. Moreover, the appended peptide sequence (AANDENYALAA) targets the truncated protein product for degradation by the Clp protease system. When the stalled ribosome is released, the 3′ end of the mRNA is open to attack by exonucleases. There is evidence that the tmRNA-SmpB complex may recruit RNase R to target the mRNA directly [69,70]. All in all, trans-translation is a remarkably elegant solution to a multifaceted biochemical problem.
While tmRNA is the principle system for ribosome rescue in bacteria, deletion of tmRNA is typically not lethal [71]. In the absence of tmRNA, one of two back-up rescue systems takes over, depending on the bacterial species [72]. In the first system, the alternative ribosome-rescue factor A (ArfA) protein binds within the open mRNA channel to facilitate recruitment of RF2 to truncated mRNA complexes [73,74]. Peptide release and recycling then are effectively promoted by the canonical factors. In _E. coli_, ArfA itself is produced from a truncated mRNA lacking a stop codon such that the protein product is normally targeted for decay by tmRNA [75]. However, when the cell’s capacity to rescue ribosomes with tmRNA is overwhelmed, ArfA can accumulate as nascent chains are released by other ArfA molecules in a positive feedback loop. In the second system, ArfB is a protein composed of two domains, one homologous to the GGQ domain of RF2 and the other a positively charged C-terminal tail [76,77]. The GGQ-containing domain binds in the peptidyl transferase centre to promote peptide release, while the C-terminus binds in the open mRNA channel of ribosomes needing rescue [78]. All three bacterial ribosome rescue pathways thus appear to similarly distinguish stalled from actively translating ribosomes through the identification of an open A site that typically lacks intact mRNA sequence. The activity of ArfA or ArfB alone appears to be adequate for viability though merely releasing the nascent peptide may be less optimal than tagging the protein for decay. Together, these three pathways provide different mechanisms for releasing ribosomes from truncated mRNAs, a function clearly essential to bacterial survival [71].

mRNAs lacking a stop codon are not the only challenge for bacteria; more subtle translational delays must also be detected and dealt with. Some pauses, for example, may never be resolved spontaneously or by EFP. If too many pauses accumulate, cells will suffer as the pool of available ribosomes is depleted. Early studies described an endonucleolytic activity that targets the A-site codon when ribosomes are stalled [79]. Once the mRNA is cleaved, the ribosomal complex becomes an ideal substrate for tmRNA and the other alternative rescue factors. Indeed, biochemical data indicate that tmRNA and ArfA act far more quickly on complexes with little or no mRNA downstream from the P-site codon [80,81]. Moreover, recent _in vivo_ data suggest that tmRNA can act on ribosomes carrying messages degraded just up to the 3’ boundary of the ribosome but without A-site mRNA cleavage _per se_ [82]. In another study, depletion of specific amino acids led to ribosome pausing and recruitment of tmRNA even in the apparent absence of any mRNA cleavage [83]. These latter studies raise the possibility that there are other signals in bacteria that allow stalled ribosomes to recruit rescue factors without prior mRNA decay; in theory, these signals could work by altering the dynamics of the mRNA channel, allowing the mRNA to simply move out of the way to accommodate rescue factors. We note that in every case where tmRNA (or ArfA/ArfB) has been demonstrated to play a role in rescue, the A site must have been accessible, at least intermittently. If we reflect on what we think of as regulated translational stalls, which are not rescued, such as in SecM or TnaC, we know that the A site is stringently occupied by aminoacyl-tRNA or release factors, respectively, bound in a non-reactive state. We suggest that some combination of the accessibility of the A-site tRNA binding site and the mRNA channel must drive the rescue response in bacteria.

Finally, there is an additional rescue-like event in bacteria wherein premature termination occurs when ribosomes have two or more consecutive codon/anticodon mismatches in the P and E sites; we have referred to this process as post-peptidyl transfer quality control [84]. We proposed that this quality control mechanism imposes an additional layer of fidelity on translation. Complexes with a double-mismatch (in which the E and P site tRNAs do not pair correctly with their respective codons) can be generated by frame-shifting or by iterated miscoding events. In these situations, RF2 is recruited to the ribosome even when a sense codon is found in the A site and terminates protein synthesis prematurely. This process does not require specialized factors but relies solely on the promiscuous activities of canonical release factors. Importantly, as the mRNA is not defective in these cases, there is no necessary connection between this premature termination event and mRNA turnover; the next ribosome may very well translate the same message correctly. As this system was only...
recently discovered in *E. coli*, it is not yet clear how broadly it is utilized in the bacterial kingdom. There is no evidence that a clearly analogous system functions in eukaryotic cells to increase the overall fidelity of translation [85].

8. Eukaryotic crisis rescue

In eukaryotes, stalled ribosome complexes on truncated mRNAs are recognized by a set of termination factor-related proteins known as Dom34:Hbs1 in yeast and PELOTA:HBS1L in higher eukaryotes (figure 3). Truncated mRNAs are generated by endonucleolytic cleavages upstream of a triggering stalled ribosome by an unknown nuclease in a process referred to as No-Go-Decay [86] or more generally as the decay intermediates of normal 3′ to 5′ exonucleolytic decay. Following endonucleolytic cleavage, the downstream mRNA fragment is degraded by Xrn1, a 5′-to-3′ exoribonuclease, and the downstream mRNA fragment is mediated of normal 3′ to 5′ exonucleolytic decay. Following endonucleolytic cleavage, the downstream mRNA fragment is degraded by Xrn1, a 5′ to 3′ exonuclease responsible for much of the RNA turnover in eukaryotic cells. The upstream fragment is targeted for decay by 3′ to 5′ exoribonucleases, in particular Skl7 and the Ski exosome complex; importantly, the rapid decay of the upstream fragment requires the repeated dissociation of stalled ribosomes by Dom34:Hbs1 as the population continues translating to the end of newly truncated mRNAs.

Eukaryotic ribosomes found on truncated mRNAs are dissocia
ted in a reaction promoted by the interface-stabilizing Dom34 protein and the ATPase Rli1 [14–16,87]. This reaction resembles the termination and recycling reactions promoted by eRF1:eRF3 and Rli1. Dom34 is homologous to eRF1 but lacks both the stop-codon recognition motif and the catalytically active GGQ motif that facilitates peptide hydrolysis. Hbs1 is homologous to the GTPase eRF3 that delivers eRF1 into the A site. Dom34-promoted subunit dissociation is codon-independent and does not involve peptide hydrolysis [87]. Biochemically, Dom34:Hbs1 (and PELO:HB51L) functions with maximum kinetic efficiency on ribosome complexes containing 3′-terminally truncated mRNA species [14,15]. The product of the Dom34:Hbs1/Rli1-mediated dissociation reaction is probably free 40S subunits and peptidyl-tRNA–bound 60S subunits.

These biochemical analyses and the homology of Dom34:Hbs1 with eRF1:eRF3 fit nicely with structural studies. Cryo-EM studies on Dom34:Hbs1 reveal that these factors bound analogously to eRF1:eRF3 [88,89], but interestingly, the N-terminal extension of Hbs1 binds in the empty mRNA channel; this latter observation provides a structural basis for the specificity of these factors for ribosomes stalled on truncated mRNAs. Cryo-EM structures of Rli1 bound to ribosomes with either Pelota or eRF1 also reveal similar modes of binding consistent with equivalent modes of action to promote subunit dissociation [89,90].

In addition to the biochemical and structural data, ribosome profiling experiments have established that Dom34:PELO and Hbs1/HBS1L are key factors for dissociating ribosomes that are trapped on truncated mRNA species in the cell [27]. These targets have been straightforward to characterize in vivo using a modified ribosome profiling approach that focuses on short footprints (16 nts) characteristic of ribosomes running to the end of a truncated mRNA. These studies show that ribosomes accumulate on short mRNA footprints specifically in a Dom34-deletion strain. The Hacl1 gene provides a beautiful example of an endogenous truncated mRNA species (generated by the endonuclease Ire1) where ribosomes accumulate in a dramatic fashion [27]. As is characteristic of No-Go Decay (NGD), ribosomes also accumulate at secondary sites upstream, where the mRNA is repeatedly cleaved by an unknown endonuclease. Iterated cleavage events lead to a ladder of RNA decay products in these Dom34-deletion strains, probably reflecting the positioning of ribosomes stacked behind the leading stalled ribosome [91]. This scenario probably explains why Dom34:Hbs1 activity has also been implicated in the case where ribosomes translate the poly-A tail and synthesize polylysine, a phenomenon often referred to as non-stop decay (NSD) [92,93]. Again, ribosome profiling has revealed a strong signature wherein Dom34:Hbs1 rescues ribosomes that are stalled on endonucleolytically cleaved mRNAs behind a leading stalled ribosome (R Green 2016, unpublished data). In all of these situations, Dom34:Hbs1 rescues ribosomes stalled on truncated mRNA.

Finally, ribosome profiling experiments revealed an unanticipated activity of Dom34:Hbs1 on ribosomes scanning (but not translating) the 3′ UTR and largely localized in the poly-A tail [27]. We have suggested that these represent scanning ribosomes that released their polypeptides properly at the stop codon and, due to modest failures in recycling, continue to scan the 3′ UTR as intact 80S ribosomes. Here, we suspect that the state of a ‘scanning’ ribosome is such that the A site is largely unoccupied.

To connect these many observations, we argue that Dom34:Hbs1-mediated ribosome rescue is simply triggered by empty A sites. As additional evidence in favour of this model, we note that the Dom34-rescue signature is distinct when amino acids or tRNAs are depleted from cells, where the former case is expected to result in an occupied A site and the latter in an unoccupied A site. For example, in our ribosome profiling studies of yeast strains lacking Dom34, we observed that 3-AT treatment led to ribosome pausing genome-wide at His codons, but that these paused ribosomes were not recognized as substrates by Dom34:Hbs1 [27]. 3-AT treatment depletes the concentration of His, probably leading to accumulation of decylated tRNA^His that binds in the ribosomal A site, precluding Dom34 binding. We suggest that tRNA depletion, by contrast, leaves the A site under-occupied and therefore responsive to Dom34. This possibility is corroborated by findings in mice where pauses induced by deficiencies in a brain-specific tRNA are rescued by PELOTA and GTPBP2, a homologue of HBS1L; rescue takes place in this system because the A site is under-occupied by tRNA [94]. Much of the literature in this area can be rationalized by thinking about the importance of A-site occupancy for Dom34-mediated rescue. Such a model is consistent with rescue pathways in bacteria (tmRNA, Araf and Aarb) that also depend on accessibility of the A site and mRNA channel for achieving selectivity.

While Dom34:Hbs1 clearly acts on mRNAs truncated upstream of the primary stalling site, it remains unclear how the leading or triggering ribosomes are rescued. For example, pauses at CGA-encoded Arg repeats in yeast induce an No-Go-Decay response in a Dom34-independent manner [95]. Going forward, achieving a full understanding of eukaryotic ribosome stalling and rescue will require clarification of the differences between translational pausing motifs and the rescue responses that they provoke. Given that rates of translational elongation are heterogeneous and under the regulation of numerous factors, it is plausible to assume that multiple mechanisms to discriminate between ribosome pausing are needed. Characterization of the kinetics of these various stall signals is an important subject left to be elucidated.
A particularly relevant biological example in eukaryotes is ribosome stalling on poly-A sequences that are abundantly generated by premature polyadenylation [96]. Previous studies have argued that poly-lysine may trigger the NSD rescue response through interactions with the rRNA-lined, negatively charged exit channel of the ribosome [97]. Work from our group has argued that poly-lysine is not sufficient (though is probably contributory) to trigger the response, but that the unusual biophysical properties of homopolymeric poly-A sequence are the primary determinant of ribosome stalling [98]. More specifically, studies in yeast and in *E. coli* show that iterated lysine sequences encoded by AAG and AAA codons behave quite distinctly from one another; poly-AAA sequences lead to unusual ribosome ‘sliding’ behaviours indicative of poor recognition of the AAA codon in the A site by the anticodon of Lys-tRNA. Sliding results in uncontrolled movement of the ribosome on these mRNAs and may allow a rescue response by systems searching for an open A site. We speculate that this situation might allow Dom34:Hbs1 to release leading ribosomes that have translated into the poly-A tail, as well as the upstream ribosomes arrested on cleaved transcripts (R Green 2016, unpublished data).

At this stage, how Dom34-independent pauses are recognized is poorly elucidated. Only a handful of factors are known to function early in the recognition process and their functional interactions with Dom34:Hbs1 are still unclear. These factors include the ribosome-associated scaffold protein ASC1/Rack1 [99] and the E3 ubiquitin ligase Heli2 [100]. Additionally, a non-proteasomal K63 polyubiquitin signal plays a critical role in this recognition step [101]. Importantly, these factors act specifically on elongating ribosomes, suggesting that extra layers of checkpoints are needed to determine aberrant pausing in elongation. Some of these factors are reported to strongly induce degradation of nascent protein but hardly affect the stability of mRNA, while others probably act earlier in the cascade of events and affect both mRNA and protein levels more equivalently. What seems clear is that multiple types of problematic translation complex converge on a related set of factors to implement protein and mRNA degradation. To further sort out how distinct inputs lead to similar outcomes, the field will be well served by biochemical functions of these factors remain to be determined and reconciled with their *in vivo* genetic phenotypes. Ribosome profiling will inevitably provide a bridge between *in vitro* mechanistic studies and broader-studied *in vivo* genetic approaches.

How then is the incomplete protein product targeted for degradation on these terminal ribosome complexes? Following subunit splitting by Dom34:Hbs1:Rli1 (PELO:HBS1:ABCE1), degradation of the aberrant protein product is achieved through ubiquitination of the nascent chain by the E3 ligase Ltn1 [102]. Ltn1 binds the large ribosomal subunit near the site where the nascent polypeptide exits and forms a complex with a series of factors including Rqc1, Rqc2 (Tae2), Npl2, Ufd1 and Cdc48 [100]. In this ribosome quality control complex (RQC), Tae2 and the other factors direct a wholly unanticipated mRNA-independent incorporation of alanine–threonine-rich peptide tags (CAT-tails, for carboxy-terminal Ala and Thr extensions) onto the truncated protein product [103]. One model is that the addition of CAT-tails leads to the exposure of lysine residues that might otherwise be sequestered in the ribosome exit channel. This level of sophistication is reminiscent of the bacterial tmRNA system that tags incomplete proteins with a ClpX protease recognition sequence. The molecular mechanism of this fascinating system is still being deciphered but promises to yield new and interesting biological insights.

### 9. Conclusion

Ribosome and protein homeostasis are critical for life in all organisms. To maintain homeostasis, elaborate surveillance systems have evolved to recognize aberrant translation, to target incomplete proteins for decay and to rescue stalled ribosomes. Interestingly, these systems must have evolved after the divergence of bacteria and eukaryotes because these systems have little in common beyond their recognition of related aberrant events. Ongoing biochemistry, genetics and ribosome profiling will continue to decipher the signals that lead to ribosome pausing or arrest, and, in turn, result in either recovery or rescue.

**Competing interests.** We declare we have no competing interests.

**Funding.** The authors’ research is supported by NIH grant nos. GM110113 (to A.R.B.) and GM059425 (to R.G.) and by the Howard Hughes Medical Institute (R.G.).

**Acknowledgements.** Thanks to Kazuki Saito for critical comments on the manuscript.

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