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Ribosome-based quality control of mRNA and nascent peptides

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Abstract

Quality control processes are widespread and play essential roles in detecting defective molecules and removing them in order to maintain organismal fitness. Aberrant mRNA molecules, unless properly managed, pose a significant hurdle to cellular proteostasis. Often mRNAs harbor premature stop codons, possess structures that present a block to the translational machinery, or lack stop codons entirely. In eukaryotes, the three cytoplasmic mRNA-surveillance processes, nonsense-mediated decay (NMD), no-go decay (NGD) and non-stop decay (NSD), evolved to cope with these aberrant mRNAs, respectively. Non-stop mRNAs and mRNAs that inhibit translation elongation are especially problematic as they sequester valuable ribosomes from the translating ribosome pool. As a result, in addition to RNA degradation, NSD and NGD are intimately coupled to ribosome rescue in all domains of life. Furthermore, protein products produced from all three classes of defective mRNAs are more likely to malfunction. It is not surprising then that these truncated nascent protein products are subject to degradation. Over the past few years, many studies have begun to document a central role for the ribosome in initiating the RNA and protein quality control processes. The ribosome appears to be responsible for recognizing the target mRNAs, as well as for recruiting the factors required to carry out the processes of ribosome rescue and nascent protein decay.

Introduction

Cells rely on a number of template-dependent processes to maintain and decipher the genetic code. These processes are among the most accurate in biology highlighting the importance of ensuring that the sequence of protein products is a faithful interpretation of the genetic information. Even with the remarkable level of accuracy of these processes, as many as one in ten newly synthesized proteins have been estimated to contain at least one miscoded amino acid [1]; most are the result of translational errors [2]. Furthermore, mRNA molecules are subject to constant changes and modifications [3, 4] that could potentially have adverse consequences on proteostasis. Defective protein products are more prone to misfold and sometimes have dominant-negative effects [5, 6]. As a result, it comes as no surprise that organisms evolved a number of quality control processes to detect errors in the mRNA and protein pools and subject them to rapid degradation [7]. Recently, it has been appreciated that the failure to elicit these quality control processes is likely to be responsible for a number of diseases and is critical for cellular fitness [8, 9].

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The biogenesis of an mRNA is complex and involves some of the most elaborate machinery in the cell, particularly in eukaryotic organisms. A mistake could result in a defective mRNA that is not true to its encoded sequence. At a minimum, transcriptional errors by the polymerase, although infrequent at a rate of about 10^{-5} [10], can result in a nonsense mutation that can lead to the translation of truncated protein. Since many mRNAs are made from a single gene, at first glance such a mutation might seem inconsequential. However, considering that some transcripts have been estimated to produce more than a thousand copies of proteins per mRNA copy [11], the cost of a nonsense mutation is not trivial especially given the expense of protein synthesis (approximately 2000 ATP molecules per one protein molecule) [12]. In addition to its cost, a truncated protein product is more likely to malfunction, adversely affecting the function of the cell [1, 6]. It should be noted, however, that more often a nonsense mutation is introduced to the mRNA molecule during processing events, particularly during splicing [13]. In eukaryotes, these mRNAs are subject to quality control through nonsense-mediated decay (NMD) [14].

Misprocessing of pre-mRNA often results in premature polyadenylation leading to truncated transcripts [15–18]. mRNA is also subject to alterations and modifications post-synthesis with potentially-profound effects on their function [4]. A key issue for maintaining the integrity of transcripts is the inherent chemical instability of the phosphodiester backbone of RNA that makes it susceptible to endonucleolytic cleavage [19, 20]. Truncated transcripts, regardless of the source, often lack a stop codon causing the ribosomes to run to the end of the mRNA and stall. These mRNAs are rapidly degraded through a process termed non-stop decay (NSD) [15, 17]. In addition to cleavage, RNA is also susceptible to chemical insults that modify the nucleobase interfering with the codon-anticodon interaction [3, 21]. Frequently these modifications on the mRNA stall the decoding process and cause the ribosome to “not go”. Certain RNA structures such as hairpins and pseudoknots also stall the ribosome [22]. No-go decay (NGD), which shares many features with NSD, is responsible for recognizing these aberrant RNAs and targeting them for degradation [23]. Notably, NGD and NSD are intimately linked to the arguably more important function of ribosome rescue [24, 25]. Indeed, initial studies on NGD and NSD focused on these two aspects of mRNA degradation and ribosome recycling [23]. However, recent studies have also begun to address the fate of the truncated protein product [26]. The defective nascent protein has been found to be the target of a quality control process that rapidly degrades it [27].

Since defective mRNAs interfere with translation, the corresponding mRNA- and protein-quality-control processes all take advantage of the ribosome for the recognition [23]. In this review, we discuss our current understanding of the mechanism of this recognition process and the initial recruitment of different quality control factors to the ribosome. We also highlight some of the gaps in our understanding of the processes and potential future investigations for the field.

mRNA-surveillance

The interplay between translation and quality control

Because mRNA-surveillance and quality control of nascent proteins take advantage of changes to the translation cycle, it is worthwhile to briefly discuss the key steps of protein

synthesis. The translation process is divided into four stages: initiation, elongation, termination and recycling. Initiation is the process by which the ribosome recognizes the start codon to begin protein synthesis. This process is different between eukaryotes and bacteria. Initiation is relatively complex in eukaryotes and is subject to various aspects of quality control, which are beyond the scope of this review. Instead a more detailed review of initiation can be found here [28].

The elongation phase is essentially the same in all domains of life [29], and it involves the recruitment of a ternary complex of aminoacyl-tRNA (aa-tRNA), elongation factor EFTu/eEF1A (bacteria/eukaryote, respectively) and GTP to decode the A-site codon (Fig. 1). EFTu/eEF1A is a member of translational GTPase factors that interact with a conserved region on the large subunit [30]. As we shall see later, among these factors are proteins that are directly involved in initiating quality control on the ribosome. The speed of the decoding process, as it happens during the elongation phase, is sensitive to many parameters which include: 1) the identity of the A-site codon as well as its sequence context [31, 32]; 2) the concentration of the corresponding aa-tRNA [22, 33]; 3) the structure of the mRNA downstream [22]; 4) the sequence of the nascent peptide and its interaction with the exit tunnel of the ribosome [34, 35]; 5) chemical modifications to the nucleotides [3]. In eukaryotes, significant decreases in the rate of peptide-bond formation, initiated by these features in the mRNA, are recognized as stalls and elicit NGD. The process is linked to ribosome disassembly and recycling of the subunits (discussed below).

Although the molecular details of the termination and recycling phases differ between bacteria and eukaryotes, the processes appear to be intimately coupled to each other in both domains of life [36, 37]. Peptide release is initiated when a stop codon arrives at the A site of the ribosome; this in turn is recognized by class I release factors RF1/2, eRF1 and aRF1 in bacteria, eukaryotes and archaea, respectively [38, 39]. In eukaryotes, similar to aa-tRNA, eRF1 is thought to form a ternary complex with a translational GTPase eRF3 and GTP, which collectively bind the A site [40]. The hydrolysis of GTP by eRF3 appears to occur after stop-codon recognition and induces a conformational change on eRF1 allowing it to engage the active site of the ribosome and promote hydrolysis of the peptidyl-tRNA [40–43]. GTP hydrolysis also promotes the dissociation of eRF3, which in turn opens up a site for the ABC-type ATPase ABCE1 (Rli1 in yeast) to bind and initiate the recycling phase. Structural and biochemical studies have provided a working model for the mode of action of ABCE1/Rli1 [37, 41, 44–46]. Like other ABC proteins [47], the factor uses ATP binding and hydrolysis to trigger a “power stroke” to change its conformation [41]. The conformational switch appears to induce ribosome dissociation through disruption of intersubunit bridges and/or further conformational changes to eRF1. Additionally in vitro experiments suggest that ABCE1/Rli1 is directly involved in peptide release since its addition to a termination reaction significantly accelerated the rate of peptide release by eRF1 [37]. In contrast to its ribosome dissociation activity, ABCE1/Rli1-mediated acceleration of peptide release does not require ATP. Therefore, ABCE1/Rli1 provides a link between termination and recycling with ATP hydrolysis acting as a gate between the two processes.

As a result of the union between termination and recycling, an mRNA lacking a stop codon (referred to hereafter as non-stop mRNA) leads to ribosome stalling because the subunits

cannot be disassembled through canonical pathways. Similar to NGD targets, non-stop mRNAs have the potential of placing a significant burden on the cell as they remove ribosomes from the translating pool. The observation that cells evolved the specific process of NSD to rescue ribosomes suggests that non-stop mRNAs are frequent. Although non-stop mRNAs can result from multiple sources (for a review on this see [48]), most likely result from premature cleavage and polyadenylation during transcription [15, 16]. Non-stop mRNAs are rapidly degraded by what appears to be a combined endonucleolytic and exonucleolytic action of the cytoplasmic exosome [49]. Although NSD and NGD do not appear to involve release factors for disassembly of the ribosome, they require release-factor-like proteins in addition to ABCE1/Rli1 [37, 45]. The details of these processes will be discussed in later sections. Due to similarities between the two pathways, particularly the sensing of a stalled ribosome, the distinction between NSD and NGD has become more ambiguous recently [23].

In contrast to NSD and NGD targets, for mRNAs harboring premature stop codons, ribosomal recycling is not a problem. Although NMD is capable of discriminating between canonical and premature stop codons, the process does not utilize specialized factors to recognize the A site and instead it continues to utilize release factors to recognize the stop codon [14]. Therefore, in principle ribosome rescue ensues as it would for canonical termination. The major role for NMD is instead focused on the recognition of its target and specifying its degradation.

Non-stop decay

Similar to most mRNA-surveillance processes, NSD was first observed in yeast, for which reporter constructs lacking an in frame stop codon were shown to turn over rapidly relative to ones harboring a stop codon [15, 17]. This rapid decay of non-stop mRNAs is coupled to translation as the addition of translation inhibitors such as cycloheximide has been shown to stabilize NSD targets [15]. In addition to these observations, many studies documented a critical role for the ribosome in the initial recognition of the defective mRNAs [17, 19, 50, 51]. In particular genetic studies identified the yeast translational GTPase Ski7 to be essential for the recognition process [17, 51]. Ski7 is related to eRF3 and has been shown to interact with the ribosome [52]; it is also a component of the cytoplasmic exosome in yeast, linking the process of recognition of the non-stop mRNA to its decay [17, 51], yet so far it has been found in only a handful of yeasts [53]. In *S. cerevisiae*, Ski7 is a paralog of the NGD factor Hbs1 (discussed later); both arising from a common ancestor after a whole genome duplication [54]. Indeed, in other organisms, Hbs1 appears to fulfill the function of Ski7 in NSD. Consistent with these observations, *Ski7* deletion strains are complemented by introducing *Hbs1* from the related yeast *S. kluyveri* [54]. The molecular details of how Ski7 (or Hbs1) facilitates the initial recognition process on the ribosome are poorly understood, but as discussed in the next section, it is likely to resemble what occurs during NGD.

Early studies on NSD suggested that it is similar to the bacterial tmRNA rescue system, in which the process is triggered by a ribosome running to the 3’-end of the mRNA [17]. This is most likely to be true for truncated transcripts. However it is most likely not the case for mRNAs lacking a stop codon that have a polyA tail, which are arguably the majority of the NSD targets [55]. Although these can originate from multiple processes, most result from a

premature polyadenylation event [15, 16]. In contrast to truncated mRNAs, on these mRNAs the ribosome stalls as it decodes the polyA tail into poly(Lys) considerably short of reaching the end of the mRNA. Consistent with these ideas, internal polyA stretches are known to stall translation and elicit NGD as characterized by its hallmark endonucleolytic cleavage [33, 35, 55]. Early studies on polyA-mediated stalling suggested that it results from extensive charge-charge interactions between the positively-charged lysine residues and the negatively-charged exit tunnel of the ribosome [34, 56]. However more recent studies have argued that stalling also depends on the mRNA sequence [57, 58]. In particular, poly(AAA) sequences are more likely to promote stalling than poly(AAG) ones even though both code for the same polypeptide sequence of poly(Lys). Irrespective of the mechanism of stalling, NSD and NGD appear to be closely related to each other as, for the most part, they utilize many of the same factors and in both cases the initial step in the pathway involves a stalled ribosome.

No-go decay

As described earlier, NGD is triggered by barriers that block ribosome movement on the mRNA [22]. Genetic studies in yeast identified the factors Dom34 and Hbs1 to be important for the process. Interestingly, Dom34 (Pelota in mammals) and Hbs1 are homologs of the release factors eRF1 and eRF3 [22, 53], respectively, highlighting the profound role for the ribosome in NGD. In contrast to eRF1, Dom34 lacks the conserved GGQ motif [59–62] required to engage the peptidyl transferase center to promote hydrolysis during termination. The factor is also missing the NIKS domain [63], which is required for recognition of the stop codons. As a result, Dom34 is incapable of carrying out the release reaction. Then, what function does Dom34 carry out on the ribosome? Biochemical reconstitution experiments by Green and colleagues provided some of the first insights into this question [46]. The ternary complex of Dom34-Hbs1-GTP was shown to split the 80S ribosome into the individual subunits suggesting that these factors rescue ribosomes *in vivo*. Consistent with this idea, the splitting reaction is not dependent on the identity of the A-site codon [46]. Furthermore, similar to normal recycling, Dom34-mediated ribosome splitting is significantly more efficient in the presence of Rli1/ABCE1 [37, 45, 64]. Overall the process is very similar to canonical recycling with Dom34 and Hbs1 substituting for the role of eRF1 and eRF3, respectively [41, 65]. Collectively, these studies were the first to highlight the importance of ribosome recycling on defective mRNAs in eukaryotes; a process that long been appreciated in bacteria through trans-translation by tmRNA [66].

The initial observation that Dom34 is active on ribosomal complexes displaying any tested codon in the A site corroborates the essential notion that it can recycle any stalled ribosome regardless of the cause, but at the same time provided a potential conflict. In particular, how does Dom34 distinguish between an elongating ribosome and a stalled one? A solution to this would-be conundrum may be partly resolved by the kinetics of the processes [23]. *In vitro*, ribosome splitting is slower than peptidyl transfer [46]. Nevertheless, differences in rates cannot account for the overall specificity, mainly due to the necessity for efficient recycling *in vivo*. For instance, rate differences have to be greater than three orders of magnitude to ensure that a 1000-amino-acid-long protein is not prematurely terminated (on average). With

these reduced rates of recycling, stalled ribosomes are likely to linger and sequester even more ribosomes upstream on the transcript.

The interaction between Hbs1 and the small subunit contributes significantly to the specificity of the recognition process [65]. The N-terminal domain of Hbs1 binds in the mRNA entry tunnel. During elongation, the mRNA occupies this site and as a result Hbs1 cannot effectively bind, preventing premature dissociation of the ribosome. Biochemical experiments are in complete agreement with this model, for which the splitting activity of Dom34-Hbs1 is significantly accelerated for complexes having little to no mRNA downstream of the P site [37, 45, 64]. Interestingly, this dependency on mRNA length is reminiscent of the processes employed by bacteria to rescue ribosomes [67], even though the molecular details are quite different.

We note that the details of the steps leading to the degradation of the mRNA is beyond the scope of this review. Relevant here, however, and to the mechanism by which the NGD targets are recognized by Dom34 and Hbs1 is the observation that the defective mRNA is endonucleolytically cleaved in the vicinity of the stall site [33], generating what essentially looks like an NSD substrate. Dom34 and Hbs1 can rescue the lagging ribosomes as they run to the end of the transcript [33], as they do not harbor mRNA in the entry tunnel. The process by which the leading ribosome is rescued is not understood, though the cleavage reaction generates an uncapped mRNA, which is rapidly degraded by the 5'-3' exonuclease Xrn1 [68–71]. It is possible, as a result of its processivity, that Xrn1 may be able to displace the leading ribosome.

Nonsense-mediated decay

NMD was initially observed in yeast, for which nonsense mutations in the URA3 gene were observed to reduce the steady state levels of its transcript without affecting its rate of synthesis [72]. These observations were soon extended into other eukaryotes. In particular, Maquat et al. showed that β-globin mRNA from thalassemic patients, which contains a premature stop codon, turned over much faster relative to a nonthalassemic one [73]. The mechanism by which the cell recognizes NMD targets, even after almost four decades of study, is only partially understood [14]. The ribosome somehow distinguishes a premature stop codon from a normal one and in addition to the ribosome and release factors, genetic studies have identified the conserved factors Upf1, Upf2 and Upf3 to be important for NMD [74–76].

One of the earlier models aimed at explaining the recognition of NMD targets relies on the presence of an exon junction complex (EJC) downstream of a premature stop codon [77–79]. The EJC is a complex deposited by the splicing machinery ~24 nt upstream of exon-exon boundaries [80]. During the pioneering round of translation, where mRNAs are still bound by the nuclear cap binding protein instead of eIF4E, the EJC complex is removed by the ribosome [81]. As most transcripts contain a stop codon in the last exon, a typical premature stop codon has a signature of an EJC present downstream that cannot be removed by the ribosome. At a molecular level, the Upf proteins are proposed to bridge a connection between the EJC and the release factors [82, 83]. This connection appears to induce posttranslational modifications to Upf1 resulting in a stimulatory signal for NMD through the recruitment of RNA degrading factors [83–86]. At the same time, the connection is likely to

inhibit interactions between eRF3 and polyA binding protein (PABP) [87], thought to be key for normal termination [88–90]. Although appealing, the EJC model falls short of explaining all of the NMD targets; for instance in yeast robust NMD is observed on unspliced transcripts [91]. There are at least two competing models that have been put forward to explain target recognition during NMD. Both rely on the fact that efficiency of decay is directly related to the length of the UTR [91]. In the first model, due to the proximity between the premature stop codon and the polyA tail, interactions between eRF3 and PAB, which are critical during normal termination, are inhibited [92]. In turn the Upf proteins are allowed to bind and effectively mark the RNA as an NMD target. This model has been called into question as others have argued that mRNAs lacking premature stop codons are destabilized regardless of whether they are polyadenylated or not [93]. In an alternative model it has been suggested that Upf1 coats the 3'-UTR [94]. Therefore for long UTRs, such as those found on NMD targets the local concentration of Upf1 is much higher, distinguishing them from non-targets. In summary, while the precise recognition of a NMD target is currently ambiguous, many in the community agree that something about the mRNA sequence downstream of a premature stop codon is responsible for initiating NMD, "the faux 3'-UTR model" [95]. Future studies are needed to clarify what these signals might be. The elucidation of the signaling cascades which lead to successful recognition and degradation of aberrant transcripts are open challenges in the field of NMD.

Quality control of nascent peptides

While ribosome-based quality control processes have been typically studied in the context of the fate of the mRNA, recent studies have begun to address the fate of the nascent peptide [26]. Defective mRNAs, be it NMD, NGD or NSD targets, have the potential to code for toxic protein products that are likely to misfold or malfunction [1]. As a result, organisms from bacteria to man appear to have evolved at least one form of co-translational protein quality control process that target defective nascent proteins. By rapidly eliminating defective proteins on the ribosome, these processes ensure that potentially toxic protein products are not allowed to cause harm to the cell. As would be predicted, for defective mRNAs the trigger for these pathways is stalled ribosomes [27, 96, 97]. But beyond this similarity, the details of co-translational protein quality control vary vastly between bacteria and eukaryotes. This is in part rationalized by differences in the mRNA-surveillance pathways.

Trans-translation in bacteria: tmRNA

In bacteria, at least one protein QC pathway is known to occur on stalled ribosomes that run to the end of an mRNA [96]. These truncated mRNAs are produced through a variety of processes that include endonucleolytic cleavage, ribosome stalling, chemical insults and premature transcriptional termination. They are for the most part subject to trans-translation by tmRNA, which acts as a tRNA and an mRNA ensuring ribosomes complete the translation cycle and hence are recycled [66]. Interestingly, the role of tmRNA in translation quality control was elucidated through its participation in protein quality control and ribosome rescue. In particular, although the RNA was initially discovered in the 1970s [98], its biological function was not uncovered until the 1990s [96]. This discovery was made possible by the observation that heterologous expression of genes in E. coli often resulted in

truncated protein products that have a defined C-terminal extension [99]. This C-terminal tag was later found to be encoded by a short ORF within the tmRNA sequence, and is referred to as an ssrA (the name of the gene encoding tmRNA) tag [96]. The sequence of this tag is similar to the degradation signal used by bacterial proteases suggesting that ssrA-tagged proteins are subject to proteolytic degradation [100]. We now know that: 1) tmRNA, which is aminoacylated by alanine [101], binds the A site of the ribosome [102]; 2) the nascent peptide is transferred to tmRNA; [103] 3) translation then switches from the 3'-end of the mRNA to the ssrA-coding sequence tagging the C-terminus of the defective protein [66, 104]. Similar to aa-tRNAs, tmRNA binds elongation factor EFTu [105, 106] but also requires another protein partner, SmpB [107]. The molecule binds the A site in a quaternary complex with EFTu, SmpB and GTP.

An important question that emerged soon after the discovery of trans-translation is how the selectivity of the process is governed. During the elongation phase of translation, the specificity of peptidyl transfer is achieved by cognate codon-anticodon interactions between the mRNA and tRNA. These interactions are critical for initiating conformational changes in the decoding center that are pre-requisites for aa-tRNA accommodation into the peptidyl transferase center [108]. However, tmRNA lacks the anticodon stem loop and as a result cannot form the same sort of interactions as aa-tRNAs [109]. The first clues about the mechanism of tmRNA recognition came from *in vivo* studies showing that ssrA tagging occurred on ribosomes that either reach the 3'-end of the mRNA because they lack a stop codon or on stalled/paused ribosomes (resulting from rare codons or inefficient peptide release due to the peptide sequence or interactions between the nascent peptide and the exit tunnel of the ribosome) [96, 110, 111]. Ribosomal complexes stalled in the middle of transcripts are converted into complexes stalled on the 3'-end of the transcript through endonucleolytic and exonucleolytic degradation of the mRNA target [111–115]. Therefore, a feature that is common to all tmRNA targets is a ribosomal complex that has little to no mRNA downstream of the P site. Indeed, in vitro studies have shown that complexes with more than six nucleotides downstream of the P site are poorly recognized by tmRNA [67, 116].

Recent biochemical and structural studies have provided important clues about the molecular mechanism of the recognition process [117]. Crystal structures of the tRNA-like domain of tmRNA in complex with SmpB revealed that the complex adopts a structure similar to a tRNA with the N-terminal domain of the protein substituting for the anticodon stem loop [118]. Consistent with these observations, cryoEM reconstructions and chemical probing experiments showed that SmpB is likely to interact with A1492, A1493 and G530 residues of the decoding center of the ribosome [119, 120]. During normal elongation, the universally conserved A1492 and A1493 residues change conformation to engage the minor groove of the codon-anticodon helix [121]. The A-minor interactions are necessary to stabilize this "induced-fit" state of the ribosome [122]. However, during trans-translation the decoding center is occupied by SmpB and hence A-minor interactions cannot occur. Consistent with these observations, mutating any of the decoding center residues, which is detrimental for normal decoding [123], has no effect on tmRNA activity in a reconstituted system [124]. Nonetheless, a recent high-resolution crystal structure of *T. thermophilus* tmRNA–SmpB-EFTu complex bound to the ribosome shows that the decoding center adopts a conformation similar, with subtle differences, to that observed with normal elongation complexes [117].

Therefore, although SmpB appears to induce rearrangement of A1492 and A1493, their identities are not critical to stabilize this induced state of the ribosome. Accordingly, the following steps of GTP hydrolysis and accommodation of tmRNA are essentially identical between the two processes and depend on “domain closure” of the small subunit where the head and shoulder domains of the 30S subunit rotate towards the subunit interface.

The high-resolution crystal structure of the trans-translation complex also revealed some important aspects about the selectivity of the process. In solution, the C-terminal domain of SmpB is unstructured [125, 126], but in complex with the ribosome forms a helical structure [117]. The helical structure extends from the A site towards the mRNA-entry tunnel, making intimate contacts with the 16S rRNA. Earlier mutational analysis had shown that this region of SmpB and its ability to form an α-helical structure is critical for ssrA tagging [124, 127]. Overall, the structure revealed that SmpB cannot bind the ribosome unless it has reached the 3’-end of the mRNA because the C-terminus of the protein occupies a site that is normally occupied by the mRNA during canonical translation. The structural clash between SmpB and the mRNA ensures that tmRNA does not bind the A site, and hence prematurely terminate protein synthesis under normal conditions. We note that these studies have addressed only the initial step of trans-translation and much more is yet to be learned about the process. For example, following the first peptidyl transfer reaction, translocation has to take place to bring the tmRNA ORF into the A site of the ribosome. The new ORF has to occupy the mRNA entry tunnel where SmpB binds initially; as a result SmpB has been predicted to change conformation to allow template switching [128]. This process, by which the resume codon of tmRNA is positioned into the A site, is not understood, but appears to be dependent on key interactions between SmpB and sequence elements upstream of the ORF [129].

Eukaryotic co-translational protein quality control

In eukaryotes misfolded proteins for the most part are degraded by the ubiquitin-proteasome system [130]. The process of ubiquitin conjugation involves three classes of enzymes: E1, E2 and E3. E1 and E2 are ubiquitin-activating and – conjugating enzymes, respectively. Substrate specificity is achieved by the E3 ligases, which recognize their protein substrates and polyubiquitinate them (typically K48-linked chain) targeting them for degradation. The first reports of co-translational ubiquitination came out of studies on the quality control of the cystic fibrosis transmembrane conductance regulator (CFTR) and Apolipoprotein B100 (ApoB) proteins [131, 132]. CFTR is a relatively large protein and is prone to misfolding. In synchronized rabbit reticulocyte extracts, CFTR was observed to be ubiquitinated before the protein was completely synthesized suggesting that the protein is targeted for degradation on the ribosome [131]. ApoB, a secretory protein, was also shown to be ubiquitinated in HepG2 cells before it is fully synthesized [132]. These studies provided the first evidence that nascent proteins in eukaryotes are subject to quality control as soon as they emerge from the ribosome.

The extent to which nascent peptides are targeted for ubiquitination was initially subject to debate [133]. Some initial studies suggested as much as 30% of newly synthesized proteins are subject to degradation [134]; others argued that the number is much less (~6%) [135]. Two recent

studies, taking advantage of puromycin labeling of nascent peptides, estimated that 12–15% of newly synthesized proteins are ubiquitinated in mammalian cell culture [136, 137]. Regardless of the actual number, it is evident that a significant amount of proteins are targeted for degradation before they are complete and the extent is likely to depend on the cell type and the cellular conditions. The mechanism by which the ubiquitination machinery recognizes the broadly defined folding state of the nascent proteins is not well understood, but is likely to involve the ribosome-associated chaperone machinery. In contrast, recognition of defective protein products encoded by aberrant mRNAs has been the subject of a number of recent studies and as a result is arguably better understood [26].

LTN1 *targets aberrant peptides for ubiquitination*

Similar to studies on mRNA-quality control processes, many of the pioneering studies on co-translational protein quality control processes came out of studies in yeast using reporter constructs. Some of these initial studies by Inada and colleagues provided the first link between the two [34, 55]. In particular, protein products produced from unstable mRNAs harboring internal polyA stretches (i.e. mimicking NSD targets) were shown to be rapidly degraded by the proteasome. Genetic screens in yeast identified a set of genes that, when mutated, stabilize NSD protein products. As would be expected, most of these genes were known to affect proteasome function, but the list also included a gene encoding an uncharacterized E3 ligase *RKR1/YMR247c* [138]. This gene is currently widely known as *LTN1.* At the time, the manner in which Ltn1 may recognize its substrates was not clear, especially since the only common feature of the targets is a truncated protein product. These details started to emerge soon after the discovery by Bengston and Joazeiro showing that Ltn1 is ribosome bound and targets stalled protein products for ubiquitination [27]. Highlighting its participation in quality control of NSD protein products, *LTN1* deletion strains are sensitive to antibiotics that promote stop-codon readthrough [27]. Furthermore, mutations in the mammalian homologue Listerin have been associated with neurodegeneration [139].

The question of how Ltn1 recognizes its targets was an intriguing one. A priori, the simplest model would entail a direct association between the factor and the ribosome near the peptide exit tunnel, where it continuously monitors the status of translation speed. As soon as the ribosome stalls, the factor ubiquitinates the nascent protein. This model could easily be negated based on the relative stoichiometry of LTN1 to the ribosome; a yeast cell has an estimated 200 copies of Ltn1 relative to 200,000 ribosomes [140]. A clue into Ltn1's mode of action was made possible by the observation that inhibition of ribosome recycling by deletion of *DOM34* stabilizes NGD protein products in a non-ubiquitinated form suggesting that ribosome splitting precedes ubiquitination by Ltn1 [33]. Further clues came out of a genome-wide screen by the Weissman group, which sought to identify factors that modulate the activity of the transcriptional regulator heat shock factor 1 (Hsf1) [141]. The genetic interaction map revealed that *LTN1* shares a similar interaction network with an uncharacterized factor termed *RQC1.* Immunoprecipitation of Rqc1 purified a complex that, in addition to Ltn1, included Tae2 (Rqc2), Cdc48 and its cofactors Ufd1 and Npl4 along with the 60S ribosomal subunit. An additional study aimed at discovering genetic interactions with Ltn1 and the Ski complex identified the same set of factors [142]. Thus, Ltn1

appears to associate with a larger complex that binds the large subunit of the ribosome; this complex is now known as the ribosome quality control complex (RQC).

Role of the RQC in degrading target peptides—The next key step in understanding the mode of action of the RQC complex was assigning the role of the different factors. Initial studies showed that deletion of any of the RQC factors stabilizes protein targets [141]. Furthermore, previous biochemical experiments established that ubiquitination of the target protein is fully dependent on Ltn1 and to some extent Rqc2, but not on Rqc1 and Cdc48 [141, 142], suggesting that Ltn1 and Rqc2 are recruited initially to the 60S subunit. The recruitment of Cdc48 to the complex requires the presence of Rqc1. Beyond a direct role in facilitating proteasomal degradation of tRNA-linked polyubiquitinated protein targets, Cdc48 has also been suggested to play a direct physical role in the removal of the protein substrate from the 60S subunit [143]. In two studies from the Hegde group, reconstitution experiments in rabbit reticulocyte extracts established a more defined view of the order of events leading up to the degradation of the protein target [144, 145]. Ribosome dissociation by Pelota (the human homologue of yeast Dom34), Hbs1 and ABCE1 (the mammalian homologue of yeast Rli1) is sufficient for ubiquitination by Listerin. Subsequent binding of Listerin to the large subunit prevents its reassociation with the small subunit; cryoEM reconstruction showed that Rqc2 (or its mammalian homologue NEMF) makes contacts with the 60S subunit and the peptidyl tRNA, preventing reassociation of 60S/40S and thus allowing Ltn1/Listerin to remain bound to the large subunit [146, 147]. These observations corroborate biochemical data showing that Listerin binding to the large subunit is dependent on NEMF. Its addition was sufficient to prevent the assembly of 80S ribosomes following splitting by the NGD factors. Rqc2/NEMF has an additional newly-described function that will be discussed in detail later.

Arguably much of our understanding of the molecular details of the process has come out of structural studies; in particular recent advances in cryoEM reconstructions have painted a more focused picture of the RQC complex. In yeast these structures took advantage of the fact that Ltn1 mutants lacking the RING domain are still efficiently recruited to the ribosome but are unable to ubiquitinate the protein substrate and hence trap the RQC complex on the 60S subunit [141, 142, 147]. On the large subunit, Ltn1 adopts an elongated structure with the C-terminal RING domain, as expected, positioned near the exit tunnel of the ribosome [147]. The extended conformation allows the N-terminal domain to interact with the sarcin-ricin loop (SRL); a conserved region on the ribosome where translation factors bind. Rqc2, in addition to its interaction with Ltn1 near the SRL, occupies the 40S-binding site explaining its function in preventing association of 40S and 60S subunits. The specificity of the factor for a rescued 60S subunit versus a free one is reconciled by the observation that Rqc2 makes intimate contacts with the peptidyl tRNA in the P site, which is the product of NGD-mediated ribosome rescue; in contrast free 60S subunits have an unoccupied P site and as a result are discriminated against. CryoEM reconstructions of the mammalian complex were in broad agreement with the yeast models highlighting a similar role for NEMF in facilitating the recognition process and recruiting Listerin to the complex [146].

C-terminal tagging of nascent peptides—Perhaps one of the most interesting discoveries to be revealed by the yeast cryoEM map is the observation of an A-site tRNA with its CCA end positioned in the peptidyl transferase center suggesting that it is likely to have participated in peptidyl transfer [147]. In the absence of a 40S subunit, an A-site tRNA rapidly dissociates; however on the RQC complex, the tRNA is stabilized through interactions with Rqc2. Sequencing analysis of the RQC-associated tRNAs in the presence or absence of Rqc2 suggested that the factor might be responsible for specifically recruiting $tRNA^{Ala}_{AGC}$ and $tRNA^{Thr}_{AGT}$ to the complex. Structural data suggests that specificity for these tRNA by Rqc2 lies in a common UUIGY motif in the anticodon loop of $tRNA^{Ala}_{AGC}$ and $tRNA^{Thr}_{AGT}$. The implications of these findings were clarified through the analysis of stabilized truncated NGD protein products (i.e. in the absence of Ltn1), which were of significantly higher molecular weights in the presence of Rqc2 relative to in its absence. Careful analysis of these products revealed that the C-terminus of these higher molecular weight products was extended through the enriched addition of alanine and threonine in a nonspecific sequence. These observations suggested that Rqc2 might be responsible for mRNA-template-independent addition of alanine and threonine and hence was termed “carboxy-terminal Ala and Thr extensions” (CAT tails). The exact mechanistic details of CAT addition are yet to be understood, but at least a potential role for the process has been identified. Deletion of *LTN1* has been previously shown to induce a heat-shock response that is dependent on the presence of Rqc2 [141]. By constructing mutants that can still support clearance of the nascent peptide but not CAT extensions, the same group showed that activation of Hsf1 is dependent on the ability of Rqc2 to add nontemplated alanine and threonine amino acids [147]. How CAT tails induce a heat-shock response is unclear at the moment. However, two recent studies from the Hartl and Joazeiro groups have argued that CAT extensions result in the formation of aggregated protein products, which might serve as a signal for the heat-shock response [148, 149]. In addition, the CAT tails are likely required to extend nascent polypeptides, which do not harbor an appropriate lysine near the exit tunnel so that LTN1-mediated ubiquitination can proceed. Furthermore it is quite possible that the CAT tails may also fulfill some other function; for example it has been hypothesized that these extensions may be used to examine the functional integrity of the large subunit in case stalling occurred due to a nonfunctional ribosome and not due to a defective mRNA. Finally, it is intriguing that this system bears similarity to the bacterial tmRNA rescue system suggesting that C-terminal tagging may have been an ancient process to track incomplete proteins.

Conclusions

Despite many recent breakthroughs in the field of ribosome-based quality control, several outstanding questions remain. One of the major open questions related to mRNA surveillance pathways is how are the ribosome and associated factors able to differentiate between translational pausing and stalling? Particularly, how slow is slow enough to trigger NGD? The ability to distinguish between the two events is crucial for initiating mRNA and peptide degradation pathways, as well as ribosomal disassembly and recycling, but only under the right circumstances. For instance, programmed pausing is a common strategy utilized by the cell to ensure proteins are properly targeted and modified; these are not

typically recognized as NGD targets. The recognition of stalled ribosomes by Dom34 is potentially determined by the kinetics of its binding in the A-site when mRNA translocation is slowed, however this has yet to be confirmed. A hallmark of NGD and NSD is an endonucleolytic cleavage upstream of the ribosome; whether this cleavage is required for recruiting Dom34 is unclear. To that end, the identity of the endonuclease and how it is recruited to the mRNA are currently unknown. Additionally, the respective molecular roles of Ski7 and its paralog Hbs1 in NSD and NGD may be overlapping, but this remains to be elucidated.

Even though NMD has been studied for decades, a unifying mechanism, which allows for the identification of every premature stop codon, remains to be elucidated. The molecular details and order of signaling events between factors at the ribosome and downstream elements need to be clarified. In addition to its role in quality control, NMD has been recently recognized to be involved in the regulation of gene expression. Its role in gene expression appears to be spatially and temporally regulated, which begs the question of how the specificity of NMD is regulated. In particular, why are certain mRNAs NMD targets in certain tissues under certain conditions, while others are in different tissues under different conditions? What about NGD, can it also be co-opted to regulate gene expression?

We have only begun to learn about the RQC process and these are exciting times as more and more details emerge. Like all quality control processes discussed here, reporter constructs have been instrumental in providing critical insights into the molecular mechanics of the process. With that said, the real cellular targets of RQC remains to be identified. Out of the 10–30% of newly synthesized proteins that are targeted for ubiquitination, what fraction is RQC’s share? Genetics and structural studies identified the factors and how they interact with the ribosome, but the mechanics of the process remain to be clarified. For instance, during CAT extensions how is translocation accomplished? Given that the addition of aa-tRNAs appears to be stochastic in nature, how does it stop? And what catalyzes the hydrolysis of the peptidyl-tRNA?

These are difficult and complex questions to address and are more than likely to require a multidisciplinary approach to tackle them. Future studies are essential not only to provide additional key insights into the mechanism of these processes but also to advance our understanding of their role in cellular fitness in health and disease.

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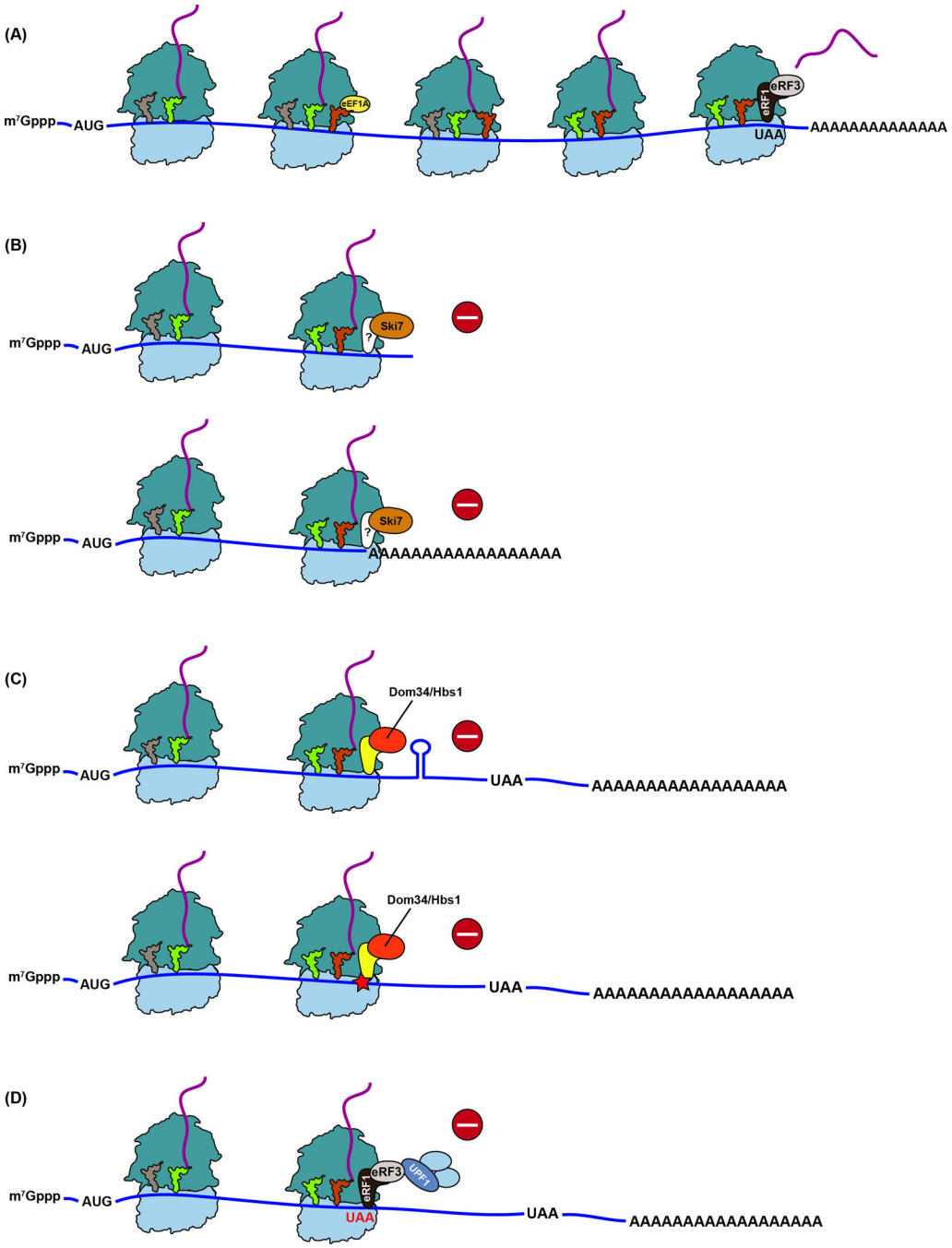

Figure 1. Translation of intact mRNAs versus aberrant mRNAs

(**A**) During normal translation, a ternary complex of aa-tRNA, eEF1A (EFTu in bacteria) and GTP binds the ribosome to decode the A site codon. Following peptidyl transfer, the elongation phase continues until a stop codon arrives at the A site, where it is recognized by eRF1 release factor in a complex with eRF3-GTP. Hydrolysis of the peptidyl tRNA and dissociation of eRF3 is triggered by conformational changes to eRF1 upon GTP hydrolysis. (**B**) In *S. cerevisiae*, the GTPase Ski7 interacts with the ribosome when it is stalled at the 3' end of a stop-codon-less mRNA (top) or when it translates a polyA tail (bottom), activating

non-stop decay (NSD). A binding partner for SKI7 has not been identified (shown as ?). (**C**) No-go decay (NGD) is responsible for recognizing and rescuing ribosomes stalled within an mRNA, either due to stable structures that block its progression (top) or caused by damaged nucleobases or strings of rare codons (bottom - shown as a star). Dom34, together with Hbs1-GTP, binds the ribosome and recycles the stalled ribosome. The process results in an endonucleolytic cleavage event (not shown), which may precede Dom34 recruitment. (**D**) Premature stop codons are recognized by canonical release factors that interact with Upf1 and other factors of the nonsense mediated decay (NMD) pathway.

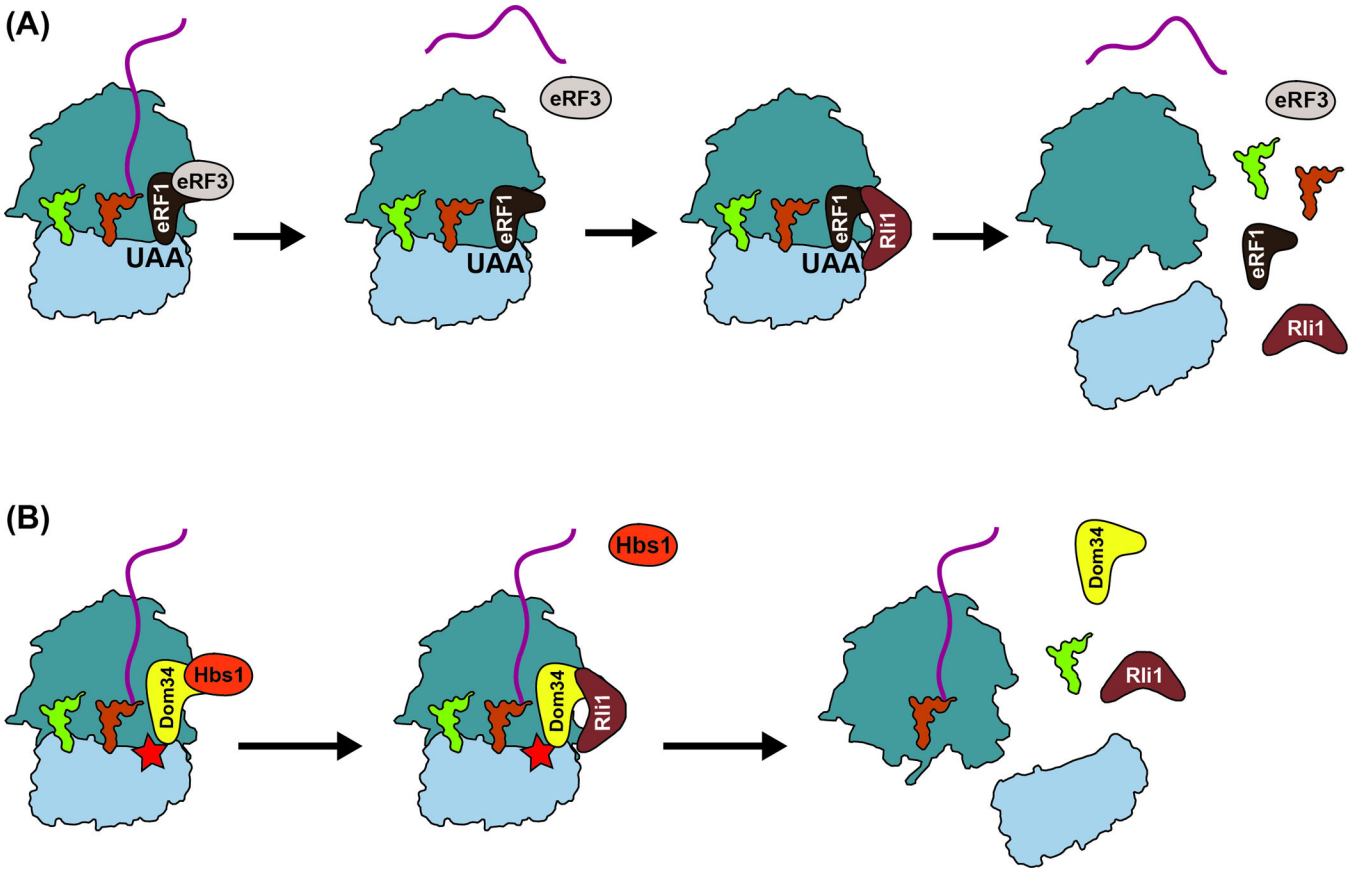

Figure 2. Ribosome recycling

(**A**) During termination under normal conditions, eRF1/eRF3 release factors recognize a stop codon and bind the ribosome. GTP hydrolysis leads to conformational changes in eRF1, which mediates release of the peptide and recruitment of Rli1. Rli1 is required to promote ribosome splitting after hydrolysis of ATP. (**B**) During NGD, Dom34/Hbs1 recognize a stalled ribosome and bind the A site. Upon GTP hydrolysis, Hbs1 dissociates, allowing interaction with Rli1 and the subunits dissociate, but without release of the peptidyl tRNA.

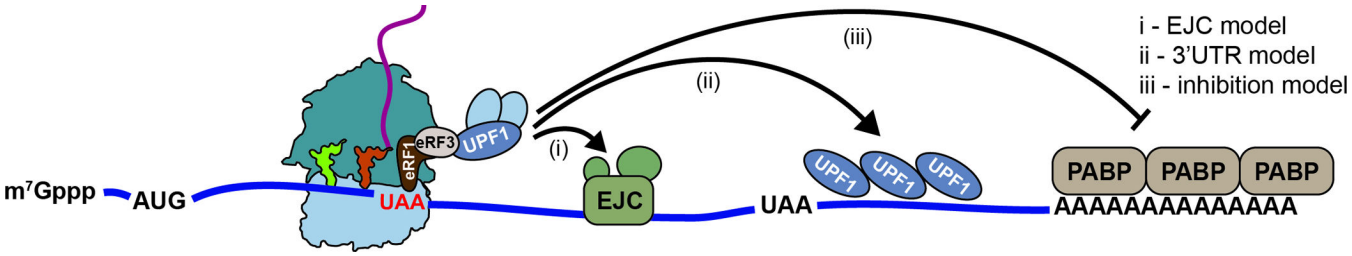

Figure 3. Models for nonsense-mediated decay

mRNAs containing premature stop codons are recognized by the cell using several possible mechanisms. (i) the EJC model relies on interactions between an EJC located downstream of the premature stop codon and the Upf proteins that are bound to release factors on the ribosome. (ii) The 3' UTR model suggests that Upf1 coats the UTR and the local concentration of the protein distinguishes NMD targets from other mRNAs. (iii) Interactions between eRF3 and polyA binding protein (PABP), essential during normal termination, are inhibited when the distance between the premature stop codon and polyA tail is large.

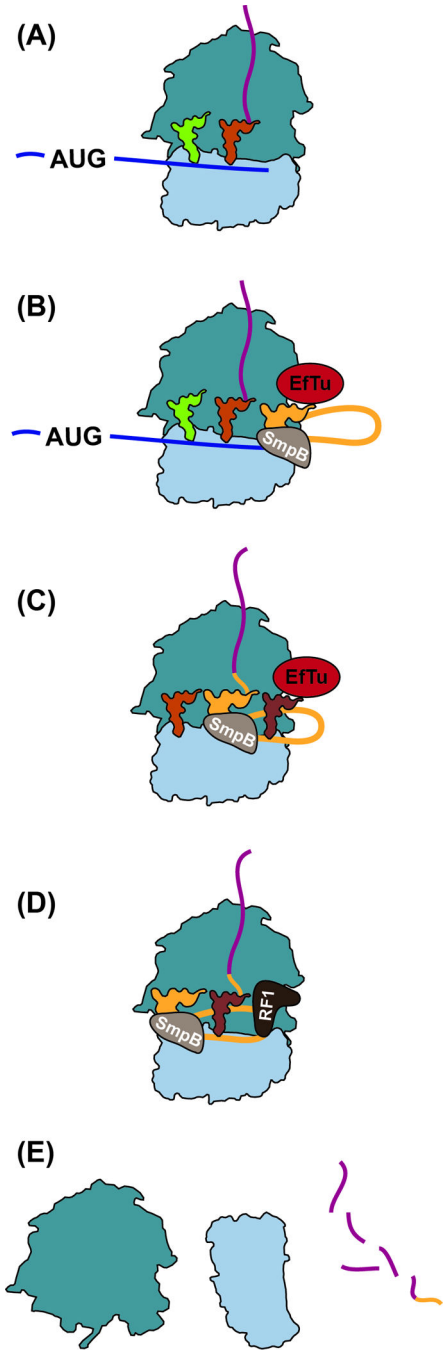

Figure 4. Ribosome rescue by trans-translation in bacteria

(**A**) Ribosomes stall at the 3' end of non-stop mRNAs or those containing rare codons. (**B**) A complex consisting of tmRNA, SmpB and EF-Tu, together with GTP, binds to the A site. (**C**) The nascent peptide is transferred to tmRNA and translation resumes on the ssrA ORF, tagging the defective protein at its 3' end. The mRNA is released and degraded. (**D**) Termination occurs on the tmRNA stop codon using standard release factors. (**E**) Ribosomes dissociate and the tagged protein is degraded.

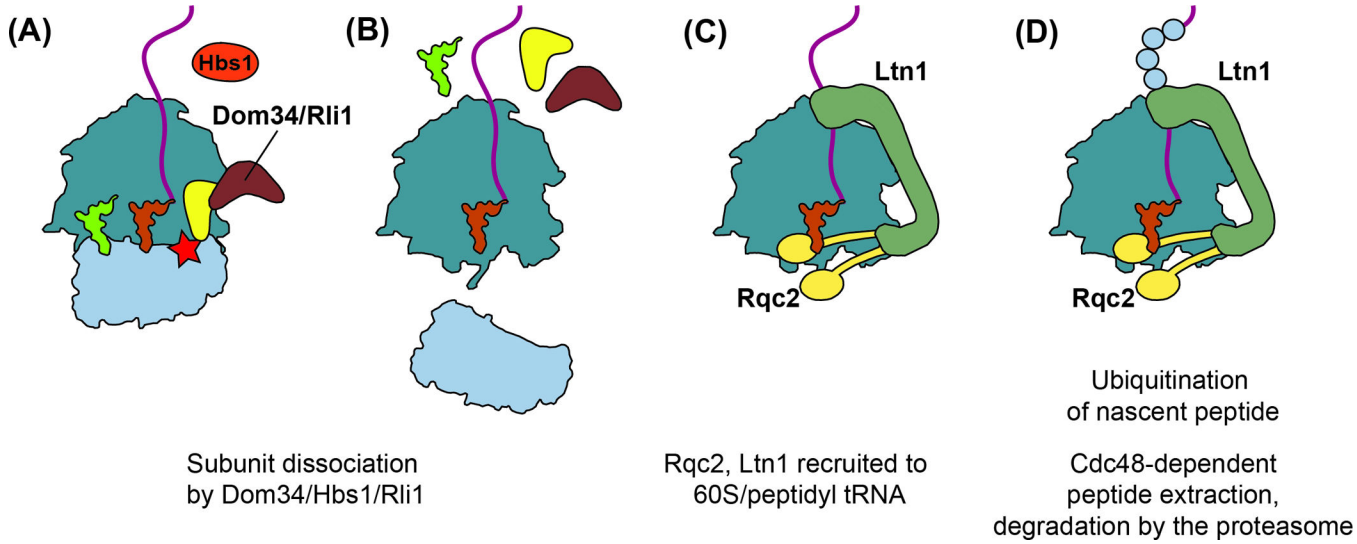

Figure 5. Co-translational protein quality control

(**A**–**B**) Ribosomes stalled on defective mRNAs are released through the NGD pathway. (**C**) Rqc2 binds the 60S subunit, contacting the exposed P site tRNA and stabilizing Ltn1 binding. The C-terminal RING domain of Ltn1 contacts the exit tunnel while the N-terminus interacts with the sarcin-ricin loop on the ribosome. (**D**) Ltn1 ubiquitinates the nascent peptide, targeting it for degradation by the proteasome. Extraction of the peptide from the ribosome depends on Cdc48.