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Molecular Highway Patrol for Ribosome Collisions

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During translation, messenger RNAs (mRNAs) are decoded by ribosomes which can stall for various reasons. These include chemical damage, codon composition, starvation, or translation inhibition. Trailing ribosomes can collide with stalled ribosomes, potentially leading to dysfunctional or toxic proteins. Such aberrant proteins can form aggregates and favor diseases, especially neurodegeneration. To prevent this, both eukaryotes and bacteria have evolved different pathways to remove faulty nascent peptides, mRNAs and defective ribosomes from the collided complex. In eukaryotes, ubiquitin ligases play central roles in triggering downstream responses and several complexes have been characterized that split affected ribosomes and facilitate degradation of the various components. As collided ribosomes signal translation stress to affected cells, in eukaryotes additional stress response pathways are triggered when collisions are sensed. These pathways inhibit translation and modulate cell survival and immune responses. Here, we summarize the current state of knowledge about rescue and stress response pathways triggered by ribosome collisions.

1. Introduction

In cells, ribosomes translate genetic information from mRNAs into proteins with the help of transfer RNAs (tRNAs). Ribosomes consist of a large subunit that contains the peptidyl transferase center, and a small subunit that harbors the decoding center (Figure 1A). During translation, the ribosome moves along the mRNA codon by codon, where each codon consists in a base triplet. Aminoacylated tRNAs enter the ribosome at the aminoacyl-site (A-site) to deliver amino acids. If the tRNA's anticodon matches the codon in the A-site, the amino acid is added to the growing peptide chain in the ribosome's peptidyl transferase center. Then the peptidyl-tRNA moves to the ribosomal peptidyl-site (P-site). The tRNA that was previously bound in the P-site moves to the exit-site (E-site) for release. Translation usually proceeds from a start codon to a stop.

1.1. Causes and consequences of ribosome collisions

Ribosomes do not always reach the stop codon as anticipated. Indeed, many problems can occur during translation, so that ribosomes stall. Trailing ribosomes can clash into stalled ribosomes, forming a collided di-ribosome (disome) (Figure 1B), or even higher order structures.[1–3] This can happen for many different reasons: mRNAs can contain roadblocks formed by stable secondary structures,[4] chemical damage, e.g., alkylation or oxidation,[5,6] misprocessing,[7,8] or unfavorable codon sequences. For example, in yeast, the CGA codon that encodes arginine is decoded very slowly. This is because the respective $tRNA^{Arg}$(ICG) has a low abundance and because base pairing between inosine and adenine is inefficient at the wobble position.[9,10] Translation of poly(A), encoding lysine stretches, is less efficient due to the specific conformation of the mRNA adopted within the ribosome, at the A-site,[10,11], inhibiting binding of aminoacyl-tRNA.[11] Additionally, the peptidyl-tRNA adopts a conformation that slows peptide bond formation.[11] Translation can also stall due to a lack of aminoacylated tRNA that in turn can be due to, e.g., a deficiency in tRNA or amino acid starvation.[12,13] Ribosomes containing faulty ribosomal RNA (rRNA) can start translating but are then targeted by different surveillance machineries, probably due to stalling.[14–16] Translation inhibitors only cause collisions when used at intermediate concentrations, high enough to stall a subset, but not all ribosomes.[3] This is because, for collisions to occur, trailing ribosomes need to continue translation until reaching a stalled ribosome.

Ribosome collisions are undesirable not only because collided complexes are unproductive, trap ribosomes and generate truncated proteins. Collisions also cause frameshifting,[17,18] where out-of-frame translation is more likely to lead to protein aggregation. Interestingly, +1 frameshifting occurs more often in yeast,[17] whereas −1 frameshifting is more common in mammals.[18] Advances in sequencing technologies have been made to identify disomes translatome-wide, exploiting the fact that disomes protect longer mRNA fragments from limited RNase digest. Such protected fragments were successfully sequenced to find that collisions occur widespread in eukaryotes, though many collisions seem to be benign and do not trigger downstream responses.[12,19–22] Indeed, ribosomes can pause for multiple reasons, including to allow for appropriate folding or necessary protein-protein interactions.[23] It is likely that necessary ribosomal pauses can also lead to collisions. If and how cells differentiate between desired pauses and undesired stalls, is subject of ongoing research, as reviewed elsewhere.[23,24]

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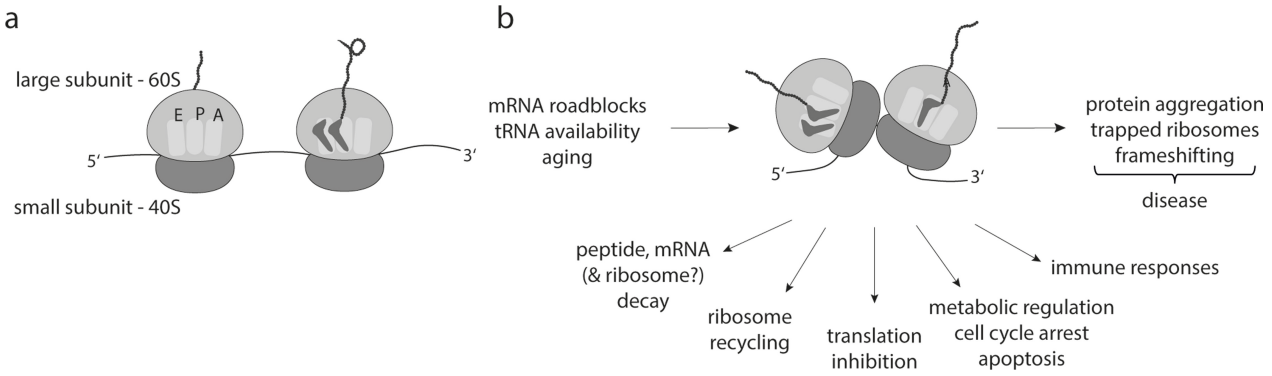

Figure 1. Translation (a) and ribosome collisions (b). A-, P-, and E-site are shown in (a), causes and consequences of ribosome collisions, as well as rescue pathways triggered upon ribosome collision are shown in (b). In collided ribosomes, the small subunits contact each other, forming a recognition platform for various sensors.

1.2. Pathways that respond to collisions

Recently, ribosome collisions (rather than simple ribosome stalls) have been found to trigger translation quality control pathways that target the different components of collided complexes. Ribosome-associated quality control (RQC) targets the incomplete nascent peptide.[3,2] Initiation RQC (iRQC) targets ribosomes that have trouble initiating,[25] while nonstop decay (NSD) and no-go decay (NGD) target the compromised mRNA.[1] Even more recently, additional cellular stress response pathways

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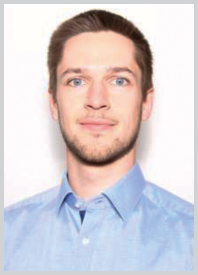

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have been discovered to respond to ribosome collisions, namely the integrated stress response (ISR), the ribotoxic stress response (RSR), and an immune response via the cyclic GMP–AMP synthase (cGAS)–stimulator of interferon genes (STING) pathway.[26–29] Diseases that have been linked to failure of quality control pathways illustrate the danger of ribosome collisions: Indeed, a number of neurodegenerative diseases in mice[30] and humans have been linked to the failure of translation quality control pathways, including Alzheimer's disease,[31] Parkinson's disease,[32] Huntington's disease,[33,34] amyotrophic lateral sclerosis and Paget disease of the bone and frontotemporal dementia.[35–37] In addition, failure of RQC has been linked to neuromuscular disease[38] and ferroptosis.[39] The process furthermore plays a role in antigen presentation.[40] Similarly, the ISR was found to be upregulated in patients with different neurodegenerative diseases, as well as generally with aging. At the same time, inhibition of the ISR leads to increased cognitive function in animals, as recently reviewed.[41] Not surprisingly, there is first evidence that excessive ribosomal pausing, as well as changes in the expression of specific translation quality control factors occur with aging[42] and contribute to the disruption of protein homeostasis. In the following, we will discuss rescue and stress response pathways of budding yeast, *Saccharomyces cerevisiae* (*S. cerevisiae*) and mammals. We will then contrast them with bacterial pathways. Figures will mostly show yeast systems. In addition to what we discuss, some research has been performed on translation quality control mechanisms in archaea[43,44] and mammalian mitochondria.[45,46] However, these will not be discussed here, in part because none of them has been linked to ribosome collisions yet.

2. Sensing Collisions and Triggering Downstream Responses of Translation Quality Control

In recent years, a growing number of proteins that are involved in sensing ribosome collisions have been discovered. Indeed, the realization that collisions are a sign of translational stress has been a major breakthrough in research concerning translation quality control. This has allowed the discovery of accessory proteins that help initiate different translation quality control pathways. Here, we will first describe sensors involved in the translation quality control pathways that deal directly with collided ribosome complexes to help initiate RQC, NSD, NGD and iRQC. Sensors involved in other pathways will be described later.

2.1. A central role for E3 ubiquitin ligases to initiate quality control

More and more evidence for mechanisms and specific players involved in sensing and removing different types of stalled ribosomes has been acquired over the last two decades. However, it has only recently become clear that ribosome collision plays a key role in recognition of translation stalling.[1–3]

In key steps towards understanding translation quality control triggering mechanisms, the ribosome-associated protein Asc1 (RACK1 in mammals)[47,48] and the E3 ubiquitin ligase Hel2 (ZNF598 in mammals; Figure 2a) were identified as essential factors for the recognition of ribosome collisions in budding yeast and human cells, respectively.[18,49–52] Asc1/RACK1 is a key

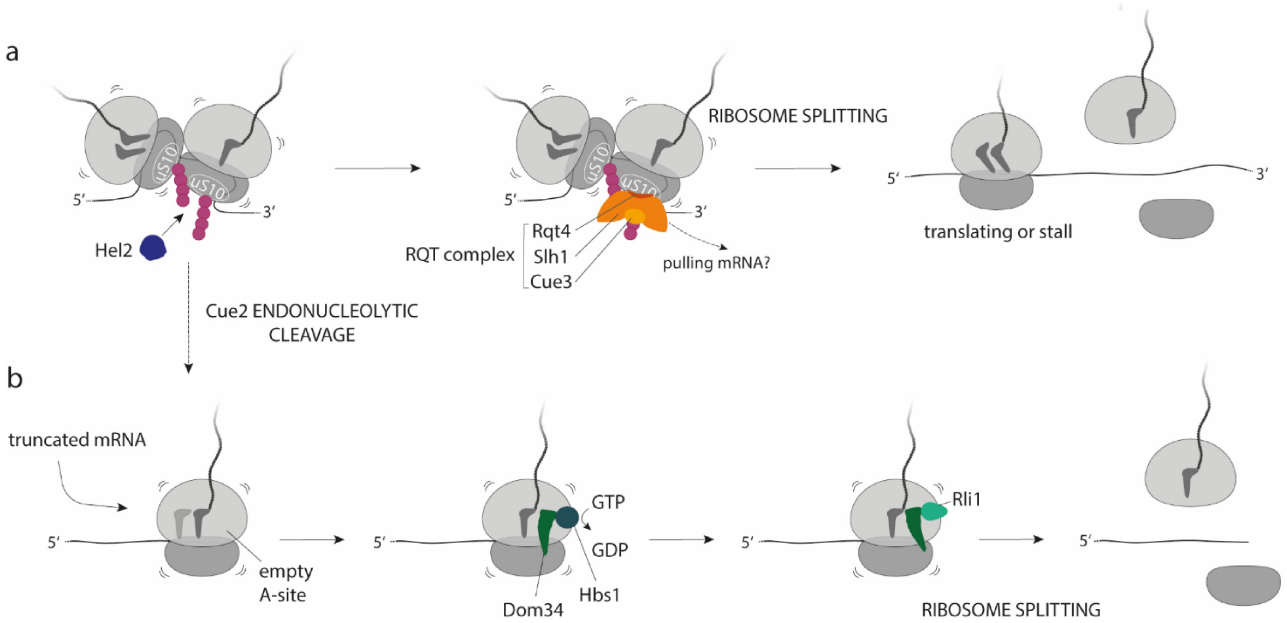

Figure 2. Ribosomal splitting factors in yeast. a) In yeast, Hel2 recognizes the 40S interface of collided ribosomes and ubiquitinates uS10. This acts as a signal to recruit the RQT complex, consisting of Rqt4, Slh1, and Cue3, to the 40S subunit of the leading ribosome and facilitates ribosome splitting. b) Ribosomes stalled at the 3′-end of cleaved or truncated mRNAs with an empty A-site are a substrate for splitting by Dom34:Hbs1:Rli1.

part of the interface formed by the small subunits of collided ribosomes which is recognized by Hel2/ZNF598.[2,3] After recognition, Hel2/ZNF598 initiates translation quality control by depositing ubiquitin marks on ribosomal proteins of the small ribosomal subunit. Here, uS10 and eS10 are the main targets in budding yeast and mammals, respectively. This ubiquitination triggers downstream responses[18,50–52] and further prevents readthrough beyond the stall site.[18,50,51] In addition, ubiquitination of ribosomal protein uS3 occurs,[18,50,51] which is dependent on ubiquitination of uS10 in yeast, but not essential for RQC.[50] Hel2 and ZNF598 harbor RNA binding sites in their unstructured C-termini[52,53] and rely on this RNA binding site for their functions in RQC and RQC-coupled NGD. Another E3 ubiquitin ligase, Not4, is important for RQC-uncoupled NGD. In this context, Not4 monoubiquitinates the ribosomal protein eS7 on small subunits of collided ribosomes. Hel2 can extend this single ubiquitin to polyubiquitin.[2,54] Collisions of initiating ribosomes in the context of mammalian iRQC are also sensed by an E3 ubiquitin ligase, RNF10, which targets ribosomal proteins uS3 and uS5.[25,55] Ubiquitination of uS5 depends on ubiquitination of uS3[56] and is responsible for degradation of 40S ribosomal subunits.[25] Despite involvement in iRQC, RNF10 has been shown to bind RNA not only close to the start codon, but across the whole RNA, with a preference for coding sequences.[55] This suggests activity not only in iRQC, but possibly also in the context of other ribosome collisions. A yeast ortholog of RNF10 exists: Mag2, which has been described to be involved in 18S non-functional ribosomal RNA decay (18S NRD).[57] 18S NRD degrades 18S rRNA - the ribosomal RNA of the small subunit - harboring a disabling mutation within the decoding center.[14] Ribosomes containing such mutated 18S rRNA can initiate translation but likely not elongate, leading to stalling at the initiation codon.[14]

In mammals, an additional E3 ubiquitin ligase, MKRN1, was found positioned upstream of poly(A) tails.[58] MKRN1 was proposed to act as a roadblock for any ribosomes that reach poly(A) tails, e.g., in the absence of a stop codon. Such ribosomes would then be ubiquitinated at eS10 to stall them indefinitely and prevent poly(A) readthrough. At the same time, this allows for recognition by collision sensors that trigger appropriate degradation pathways for the mRNA blueprint as well as resulting nascent peptides.[58]

2.2. Balance between ubiquitination and deubiquitination

While Hel2/ZNF598 and Mag2/RNF10 have been discovered as E3 ubiquitin ligases involved in triggering different branches of translation quality control, their ubiquitination activity is also counteracted by specific deubiquitinases. In mammals OTUD3 and USP21 counteract ubiquitination of uS10 and eS10 by ZNF598.[59] Ubp3 (in complex with Bre5) in yeast[60] and USP10 (in complex with G3BP1/2) in mammals[25,55] deubiquitinate uS3,[25,55,60] uS5[25,55] and uS10.[56] These activities are thought to fine-tune translation quality control, e.g., by allowing for escape from quality control and may also contribute to removing the regulatory marks on ribosomes or ribosomal subunits once collisions have been resolved.

2.3. Ribosomal splitting factors

Ribosomes trapped in collisions need to be removed from mRNAs to allow degradation of both, the mRNA and the nascent peptide. In the context of translation quality control, two different ribosomal splitting factors have been described: the RQC trigger (RQT) complex and Dom34:Hbs1 (PELOTA:HBS1 in mammals). Both complexes exhibit different target specificities. While the RQT complex targets ribosomes stalled on intact mRNA and require an accessible 3'end,[61,62] Dom34 and its interaction partners split ribosomes with an empty A-site close to the 3'-end.[12,63–66] Also, while the RQT complex is primarily associated with the RQC pathway (see 3.1), Dom34 and Hbs1 are needed for endonucleolytic cleavage in NGD and NSD (see 3.2).[4,12,62,67–70] Endogenous targets of the system would be mRNAs that have been targeted by NGD and have been cleaved by Cue2 (see 3.2).[12,68] Despite these general associations, the RQT complex is needed for RQC-coupled NGD (see 3.2) and mammalian PELOTA:HBS1 has been used to reconstitute nascent peptide chain ubiquitination *in vitro*.[71] Hence, both factors (can) play a role in both pathways.

2.3.1. The RQC trigger complex – a splitting factor for collided ribosomes

One key question was how the E3 ubiquitin ligases trigger translation quality control. To partially answer this question, in 2017, the Inada and Brandman laboratories have identified the RQC trigger (RQT) complex (Figure 2a) using mass spectrometric analyses of Hel2 co-immunoprecipitations in budding yeast.[50,72] As its name suggests, this complex can trigger RQC. In budding yeast, the RQT complex consists of the helicase Slh1, ubiquitin binding protein Cue3 and Rqt4/YKR023W.[50,72]

For mammalian cells, orthologs exists as well, namely ASCC2 (ortholog of Cue3), ASCC3 (ortholog of Slh1) and TRIP4/ASC-1 (ortholog of Rqt4).[50,73,74] Together with ASCC1, these players belong to the ASC1 complex,[74] which also helps resolve alkylative damage of deoxyribonucleic acid (DNA) in mammalian cells.[75–77] The complex has been shown in mammalian cells to perform the same role as the RQT complex in yeast: The main function of the RQT complex described is splitting of collided ribosomes.[50,62] It is Slh1 (or ASCC3 in mammalian cells) that splits the leading ribosome,[61,74,78] depending on its ATPase function.[2,50,73,74]

Splitting results in a peptidyl-tRNA bound 60S subunit and a 40S subunit. Higher-order collided polysomes are split more efficiently, in a sequential manner,[61,78] as the RQT complex requires neighboring ribosomes.[61] While the role of Slh1/ASCC3 in the RQT complex is quite well described, until recently it was less clear for Cue3/ASCC2 and Rqt4/TRIP4. Deletion or knockdown of these players individually leads to weaker phenotypes and a partial reduction of RQC,[50,72–74] whereas

double deletion leads to a similar phenotype as deletion of Slh1.[50] This suggested that both players are not essential for RQC but support the activity of Slh1/ASCC3 as modulators. A very recent report now shows that both, Cue3 and Rqt4 bind to K63-linked polyubiquitin chains on collided ribosomes, facilitating RQT complex recruitment.[79] Finally, the fourth member of the mammalian ASC1-complex, ASCC1, has no effect on ribosome dissociation.[74] A recent study[61] sheds more light on the mechanism behind ribosome splitting by the RQT complex, presenting cryo-electron microscopy (EM) structures of the RQT complex bound to collided disomes. These structures reveal that the RQT complex splits the leading ribosome in a collision because it cannot access the colliding ribosomes as it would clash with ribosomal protein subunits.[61] Furthermore, the complex requires accessible mRNA for its activity and binds to the leading 40S subunit, close to the mRNA entry tunnel. Cue3 and Rqt4 bind to and stabilize the two lobes consisting of Slh1's two helicase cassettes.[61] Interestingly, the mammalian RQT complex also interacts with ribosomes in the absence of ZNF598 and hence in the absence of ubiquitinations essential for RQC.[74] As a model for its activity, the structural work suggests that the RQT complex binds the 40S subunit of the leading ribosome around the mRNA entry tunnel and pulls the mRNA via its C-terminal helicase cassette.[61] This leads to a conformational change that destabilizes the 60S subunit. The concomitant change in the 40S subunit leads to the clash between the Asc1 (RACK1) proteins in the leading and colliding ribosome. Consequent conformational changes lead to clashes between Asc1 and the 60S subunit of the leading ribosome, causing ribosomal dissociation.[61]

Besides its role in triggering RQC, at least some subunits of the RQT complex are also needed to trigger 18S NRD.[57] Whether collisions are important here has not been elucidated. However, a role of collisions has been suggested in a related phenomenon – initiation RQC. Here, collisions, either between several scanning preinitiation complexes or between a terminally stalled ribosome and a preinitiation complex have been postulated.[25]

2.3.2. Dom34:Hbs1 complex – a splitting factor for ribosomes with empty A-sites

Besides the RQT complex, there is a second system that can help splitting of collided ribosomes: The complex of Dom34 (PELOTA in mammals) and Hbs1 (Figure 2b). The two partners are structurally similar to the canonical release factors involved in translation termination – eRF1:eRF3.[80–82] They bind to ribosomes in the ribosomal A-site with a similar binding mode.[43,63] The Dom34:Hbs1 complex forms a larger complex with the RNA exosome and the Ski complex[67] which are needed for 3'-to-5' degradation of RNA.

Compared to termination factor eRF1, Dom34 lacks the highly conserved motif which is required for stop codon recognition.[80,81] Hence, it does not require a stop codon to be present in the ribosomal A-site.[65,83] In contrast, an empty A-site[63,64] and a free mRNA entry tunnel[64] are the two requirements for Dom34 binding. Dom34:Hbs1 can therefore split ribosomes at the 3'-terminus of truncated mRNAs.[12,63–66,70] Similar to the activity of eRF1:eRF3, the Dom34:Hbs1 complex destabilizes the ribosomal subunits. Here, the ATPase activity of a third factor, Rli1 (ABCE1 in mammals) is required,[65,66,83] which is recruited upon GTPase activity of Hbs1.[66] Because Dom34 lacks the catalytic GGQ motif present in eRF1,[80,81] the peptidyl-tRNA is not cleaved from the nascent chain and therefore stays intact after ribosomal splitting.[61,65,83]

3. Translation Quality Control Pathways

Ribosome collisions elicit several responses that directly deal with the components of the collided complex, namely the nascent peptides resulting from faulty translation, the mRNA, as well as ribosomes involved.

3.1. Ribosome-associated quality control – removing unfinished nascent peptides

The removal of potentially unfinished and dysfunctional protein products arising from collided ribosomes is an important and evolutionarily conserved process known as ribosome-associated quality control (RQC; Figure 3).[49]

Because RQC has been reviewed extensively elsewhere,[84–94] we will keep its description rather short here. Splitting of collided ribosomes (discussed above) results in a free small ribosomal subunit and a peptidyl-tRNA bound large subunit. Nascent peptide degradation is catalyzed by the proteasome and depends on the RQC complex, consisting of Ltn1 (Listerin), Rqc1 (TCF25), Rqc2 (NEMF) and Cdc48 (VCP/p97; sometimes not considered as part of the RQC complex itself). Upon recognition of the 60S:nascent-peptide:tRNA complex, Rqc2 supports the recruitment of Ltn1 by stabilizing the latter on the complex.[95,96] Ltn1 poly-ubiquitinates lysine residues of the nascent chain[97] and depends on Rqc1 for this activity.[98] Rqc2 also recruits aminoacyl-tRNA to the complex and catalyzes the non-templated addition of Carboxy-terminal Alanine and Threonine (CAT) tails, also dubbed as Carboxy-Terminal tails due to a strong preference for alanine in mammals.[99,100] CAT-tailing helps Ltn1 access lysine residues still buried within the ribosomal exit tunnel[101] and possibly within less flexible 3D structures[102] for ubiquitination. CAT-tailing also requires the eukaryotic initiation factor eIF5A, potentially to stabilize the peptidyl-tRNA in the P-site during the process.[103] In addition to exposing lysines, CAT-tails may also act as degrons to facilitate nascent peptide degradation of some substrates by the proteasome, independent of Ltn1. This requires the protein Hul5 which is known to have E4 ubiquitin ligase activity, extending existing monoubiquitin moieties to polyubiquitin chains. However, in the case of CAT-tailed substrates, the deletion of Hul5 did not result in a visible mono-ubiquitinated product and further research is required to understand the preceding uncharacterized E3 ligase activity.[102] In mammals, a different system has been described that involves the E3

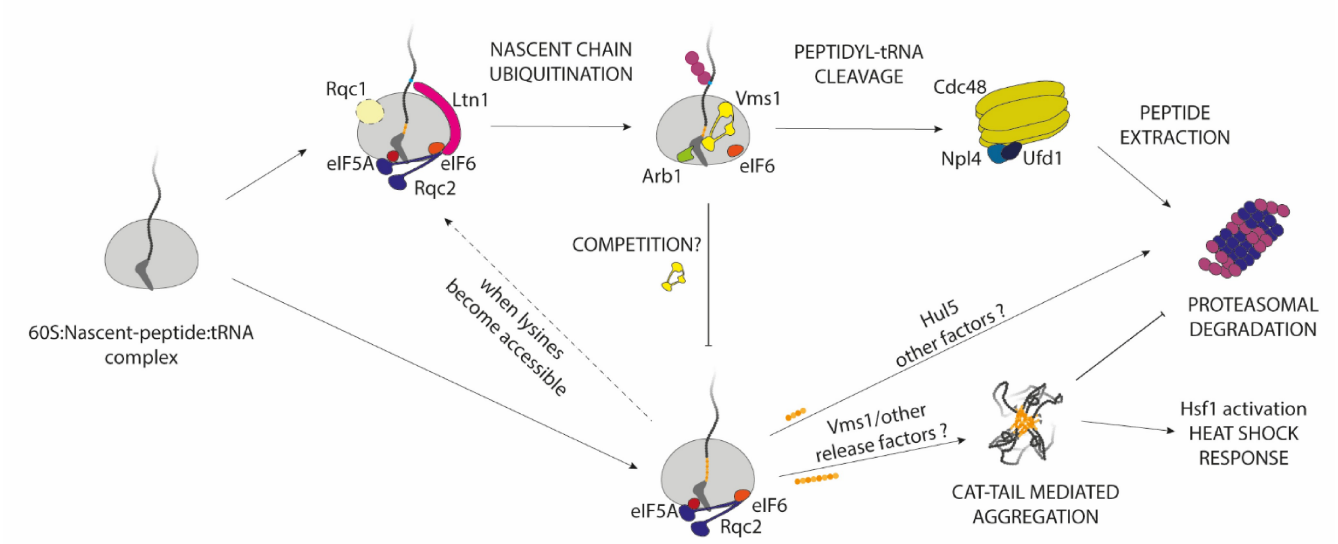

Figure 3. Ribosome-associated quality control in yeast. The 60S:nascent-peptide:tRNA complex is recognized by the CAT-tailing protein, Rqc2. (Top) Predominantly, 60S:nascent peptide:tRNA complexes are bound by the RQC complex composed of Rqc1, Rqc2, and Ltn1. The binding site of Rqc1 is unknown. Rqc2 recruits Ltn1 which ubiquitinates lysine residues (blue solid circle) on the nascent peptide near the exit tunnel. Rqc2 aids in the exposure of lysines in the exit tunnel by adding CAT-tails (light and dark orange solid circles) with the help of eIF5A. This is followed by the endonucleolytic cleavage of the peptidyl-tRNA to release the nascent peptide. The ubiquitinated peptide is recognized and extracted by the Cdc48:Npl4:Ufd1 complex and targeted for proteasomal degradation. (Bottom) In the absence of Ltn1 binding, Rqc2 continues to add CAT-tails. Controlled CAT-tailing (represented by shorter CAT-tail) may promote proteasomal degradation whereas excessive CAT-tailing (represented by longer CAT-tail) may promote aggregation of the nascent peptide, impeding proteasomal degradation and triggering heat-shock response via Hsf1. Vms1 competes with Rqc2 to inhibit CAT-tailing. Hul5 further mediates the degradation of proteins that were CAT-tailed in a controlled way.

ubiquitin ligase complex CRL2^{KLHDC10} and a newly discovered C-end rule E3 ubiquitin ligase, Pirh2/Rchy1.[100] The C-end rule pathway does not exist in yeast, whereas the ortholog of Hul5 is apparently not involved in CAT-tail degron triggered degradation in mammals.[100] This suggests that different organisms have evolved varying systems to remove CAT-tailed proteins independently from Ltn1/Listerin. Excessive CAT-tailing, on the other hand, can lead to aggregation of nascent peptides, cause proteotoxicity, and trigger the heat shock response.[49,104,105] Recent evidence suggests that this happens through activation of stress response factor Hsf1 by aggregated CAT-tails.[105]

The role of Rqc1 is not fully understood, in part because it has not been detectable in structural analyses.[103] Together with Ltn1 Rqc1 is necessary to recruit Cdc48 which extracts nascent peptides from the 60S subunit to target them for degradation.[104] This process competes with CAT-tailing, as does cleavage of the tRNA from the nascent peptide by Vms1 (ANKZF1).[106–108] Vms1 activity is understood to inhibit CAT-tailing in part by competing with Rqc2, which is especially required in cases of mitochondrially targeted substrates to prevent the formation of aggregates inside the mitochondrion.[108] Vms1 and ANKZF1 have been shown to endonucleolytically cleave the peptidyl-tRNA to release the nascent peptide.[106,107] In mammals, ANKZF1 has been shown to preferentially catalyze the cleavage of ubiquitinated nascent peptides while Ptrh1 is suggested to be required for the cleavage of non-ubiquitinated peptidyl-tRNA.[109] It is currently unknown whether Vms1 can cleave non-ubiquitinated peptidyl-tRNA and whether the probable candidate ortholog of Ptrh1, Pth2,[93] participates in the process. Consistent with this idea, Vms1 enrichment shifts from the 60S to lighter fractions in Ltn1-deficient cells.[106] Deletion of Rqc2 did not affect Vms1 binding to the 60S fraction.[106,107] This raises the question of whether the mere presence of Ltn1 in the cell is sufficient to recruit Vms1 to the 60S subunit. One study also observed that overexpressed Vms1 did not shift Rqc2 from the 60S to lighter fractions[107] in contrast to what was seen previously.[108] More research is required to resolve these inconsistencies.

3.2. Nonstop and no-go decay – removing problematic mRNAs

Different types of corrupted mRNAs cause slightly different but interconnected responses that share many of their players. Nonstop-decay (NSD) was originally discovered at the beginning of the century, as a pathway targeting mRNAs without a stop codon, where translation continues into the poly(A) tract.[7,8] On the other hand, NGD was first identified as a pathway targeting mRNAs with a strong secondary structure that constitutes a translational roadblock[4] and was indeed the first rescue pathway observed to depend on ribosome collisions (Figure 4).[1] Nonstop and no-go decay have been extensively reviewed in the past.[88,90,94,110–120] Besides stating the general principles of the pathways, we will therefore focus mostly on recent developments.

NSD relies on 3′-to-5′ degradation of mRNA via the multi-protein complex called RNA exosome and requires the Ski complex composed of Ski2, Ski3, and Ski8. The Ski complex acts

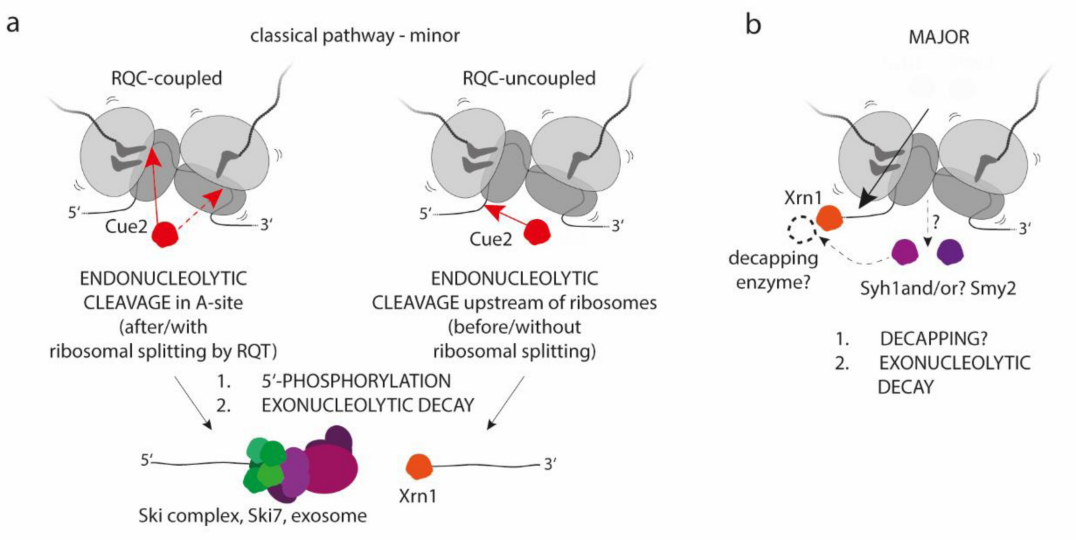

Figure 4. No-go decay in yeast. a) RQC-coupled (left) or uncoupled (right) NGD, depending on endonuclease Cue2. Cue2 cleaves mRNA endonucleolytically in the A-site predominantly of colliding ribosomes in case of RQC-coupled NGD, or upstream of collided ribosomes in case of RQC-uncoupled NGD. 5′-phosphorylation and Xrn1- and exosome-dependent exonucleolytic decay follow in both cases. b) Xrn1-dependent exonucleolytic decay. Syh1 and Smy2 might recruit Xrn1 or modulate its activity. A decapping step would likely be necessary prior to exonucleolytic decay.

as an accessory to the RNA exosome and can help unfold structured RNA.[121] Additionally, the protein Ski7 in yeast, or the short splice isoform of HBS1L in mammals is also required to provide a linkage between the Ski complex and the RNA exosome.[110,122] In addition, NSD requires Dom34:Hbs1 as a splitting factor (see 2.4).[110]

For NGD, the RQT complex is required for the classical RQC-coupled NGD pathway (Figure 4a). In the first step, it cleaves mRNAs containing roadblocks endonucleolytically. This is then followed by ribosome dissociation and exonucleolytic degradation of both cleavage products via the RNA exosome and the 5′-to-3′ exonuclease Xrn1.[4] Recently, the endonuclease has been identified as Cue2.[62] N4BP2 is the putative mammalian ortholog.[62] *Caenorhabditis elegans* has an ortholog called NONU-1 which has been experimentally verified.[123] Cue2 cleaves either within collided ribosomes, when NGD is coupled to RQC, or upstream of collided ribosomes, when NGD is uncoupled from RQC (Figure 4a).[2,54] Cleavage during RQC-coupled NGD occurs in the A-site, predominantly of colliding ribosomes[62] and requires ubiquitination of uS10 by Hel2. In yeast, RQC-coupled NGD also requires the helicase Slh1/the RQT complex (see 2.3) and factor Mbf1, depending on the type of stalling sequence.[54] In the absence of Slh1 and Mbf1,[54] cleavage occurs upstream of colliding ribosomes. Here, cleavage relies on polyubiquitination of eS7, initiated by Not4 and extended by Hel2.[2,54] As another distinctive feature, Cue2's ubiquitin binding CUE domains at its N-terminus, are important for RQC-uncoupled NGD, but dispensable for RQC-coupled NGD.[54]

The endonucleolytic cleavage during NGD in yeast results in 3′-cyclic-phosphate and 5′-OH. The latter is phosphorylated by a tRNA ligase with kinase activity to allow degradation by 5′-phosphate dependent exonuclease Xrn1.[124] Interestingly, the mammalian Cue2 ortholog N4BP2 harbors a polynucleotide kinase domain and may perform phosphorylation itself.[62] Degradation of no-go type mRNAs does not necessarily rely on endonucleolytic cleavage. Indeed, simple exonucleolytic decay via Xrn1 has been reported to constitute the main pathway of NGD: upon lack of Xrn1, the levels of a non-stalling mRNA were almost restored to the level of a non-stalling control mRNA.[62,125] In contrast, upon lack of Hel2, mRNA levels were only minimally affected or even further decreased, depending on the reporter construct.[125] Very recently, two new NGD factors, Syh1 and its paralog Smy2 have been identified.[125] Both are orthologs of mammalian GIGYF2, a factor previously shown to be involved in repressing translation.[126] GIGYF2 has also been observed to repress translation of collision-prone mRNA as further discussed in section 4.2.[127] In contrast, Syh1 and Smy2 destabilize stalling-prone mRNA. The effect of Smy2 on the stability of stalling-prone mRNA is lower than that of Syh1, but both overlap functionally.[125] While the exact mechanism is yet unknown, the recruitment of those two factors does not involve Hel2.[125] This arm of NGD also does not involve either Mbf1 or Eap1. In contrast, the mammalian orthologs GIGYF1/2 depend on the ortholog of Mbf1 (EDF1) and a protein similar to Eap1, 4EHP, see 4.2.[127]

In the absence of both, Hel2 and Syh1, mRNA levels of a stalling reporter are equal to the levels of an equivalent non-stalling reporter, suggesting no NGD occurs.[125] Besides Hel2-dependent NGD, Xrn1-mediated decay has been reported to constitute the main and only other NGD arm.[62] Therefore, Syh1/Smy2 would likely be linked to Xrn1-mediated decay (Figure 4b). Indeed, Smy2 was previously found to be associated with Xrn1.[128] However, how Syh1/Smy2 are recruited and whether they play a role in Xrn1-mediated decay still needs to be elucidated.

While NSD and NGD seemed to differ initially, both pathways share many similarities and their distinction is not always

clear. Targets for NSD in the classical sense, where translation continues into the poly(A) tract, could result from premature polyadenylation. In this event, the poly(A) tail would be added to mRNAs before the stop codon is transcribed.[110] Although NGD was first found in the context of structural roadblocks,[4] additional features were also shown to cause NGD including stall-inducing codon stretches, among them poly(A) tracts,[68] a classical NSD target. Also, endonucleolytic cleavage of NGD targets results in truncated mRNA. If translation by trailing ribosomes continues to the cleavage site, the ribosomal A-site is devoid of mRNA, generating a nonstop situation. Hence, the distinction between NSD and NGD is likely less clear than initially thought.[68]

3.3. Initiation RQC – removing ribosomes with problems during initiation

An additional quality control pathway that was recently discovered has been termed initiation RQC (iRQC; Figure 5). It targets ribosomes that have failed to initiate properly, e.g., due to the presence of translation inhibitors.[25] This pathway, only described in mammals so far, degrades small ribosomal subunits of stalled initiating ribosomes.[25,55] Collisions seem to be involved here as well, either between several scanning preinitiation complexes or between a terminally stalled ribosome and a preinitiation complex.[25] As a consequence, uS3 and uS5 of the small ribosomal subunit are ubiquitinated by the E3 ubiquitin ligase RNF10 and proteins of the small ribosomal subunit are degraded. Both, degradation of ubiquitin-marked ribosomal subunits via the lysosome[56] and the proteasome[25] have been described in conflicting reports.

Interestingly, as well, RNF10 not only binds RNA close to the start codon, but also throughout the coding sequence.[55] This observation suggests that RNF10 is either not only involved in iRQC/decay of the small subunit, or that small ribosomal subunit degradation can also be an outcome of a more classical ribosome collision.

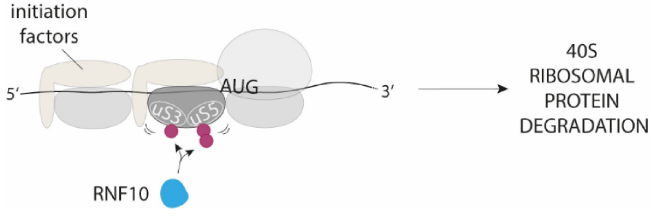

Figure 5. Initiation RQC. In mammals, collisions during translation initiation are recognized by RNF10 which ubiquitinates uS3 and uS5 and lead to 40S ribosomal protein degradation.

4. Responses Beyond Translation Quality Control

Besides direct consequences on the components of the collided complexes (ribosomes, mRNA, nascent peptides), there are additional cellular reactions to ribosome collisions that occur at the level of translation, cellular metabolism, as well as antiviral responses (Figure 6).

4.1. Activation of the ISR upon ribosome collisions

One such consequence is the activation of the ISR, which downregulates translation. This pathway can be activated by various stresses, including hypoxia, nutrient or amino acid deprivation, and unfolded proteins.[129] All these events can result in the activation of Gcn2 kinase.[130–132] In turn, this leads to phosphorylation of the translation initiation factor eIF2α.[133] As a result, 5′-cap dependent translation initiation is inhibited,[133] by preventing the exchange of eIF2-bound GDP for GTP. This suppresses the delivery of methionine-tRNA[134,135] to the small ribosomal subunit for translation initiation. While translation is generally inhibited, the expression of the transcription factor Gcn4 (ATF4 in mammals) and of amino acid genes is upregulated.[133] One of the triggers for Gcn2 activation is the presence of deacylated tRNA.[131] However, recent works show that stalled[136] or rather collided ribosomes[28] also activate Gcn2 independently of the presence of deacylated tRNAs. For this, Gcn1, which is required for Gcn2 activation,[133] binds to disomes.[27] Gcn1-bound disomes were also bound by a highly conserved complex of Rbg2 and Gir2 (DRG2 and DFRP2 in mammals[137]) at the leading ribosome.[27] Since Gir2 interacts with Gcn1 through the same domain that Gcn2 binds,[27,138,139] both proteins compete for Gcn1 interaction.[138,139] In contrast, Rbg2 interacts with the A-site tRNA on the leading ribosome and may stabilize this tRNA to promote peptide bond formation and resume translation, instead of activating Gcn2.[27]

It has been observed by several groups that the ISR is more strongly activated when the master regulator of RQC and NGD, Hel2, is missing.[19,26] On the other hand, Hel2-mediated ubiquitination is increased when the ISR is disabled. This is likely due to a lack of initiation inhibition which in turn causes an increase in ribosome collisions.[26] In cells, it is interpreted that the ISR can compensate when Hel2 becomes overwhelmed by too many ribosome collisions.

Similar to what has been described in yeast, in mammalian cells, GCN2 is also activated by collided ribosomes, dependent on its co-activators GCN1 and GCN20, as well as the MAP kinase kinase kinase (MAP3K) ZAKα, which binds to collided ribosomes.[28]

4.2. Preventing frameshifts

An additional role of Gcn1 and Gcn20, beyond the activation of the ISR has been described recently: the two factors also

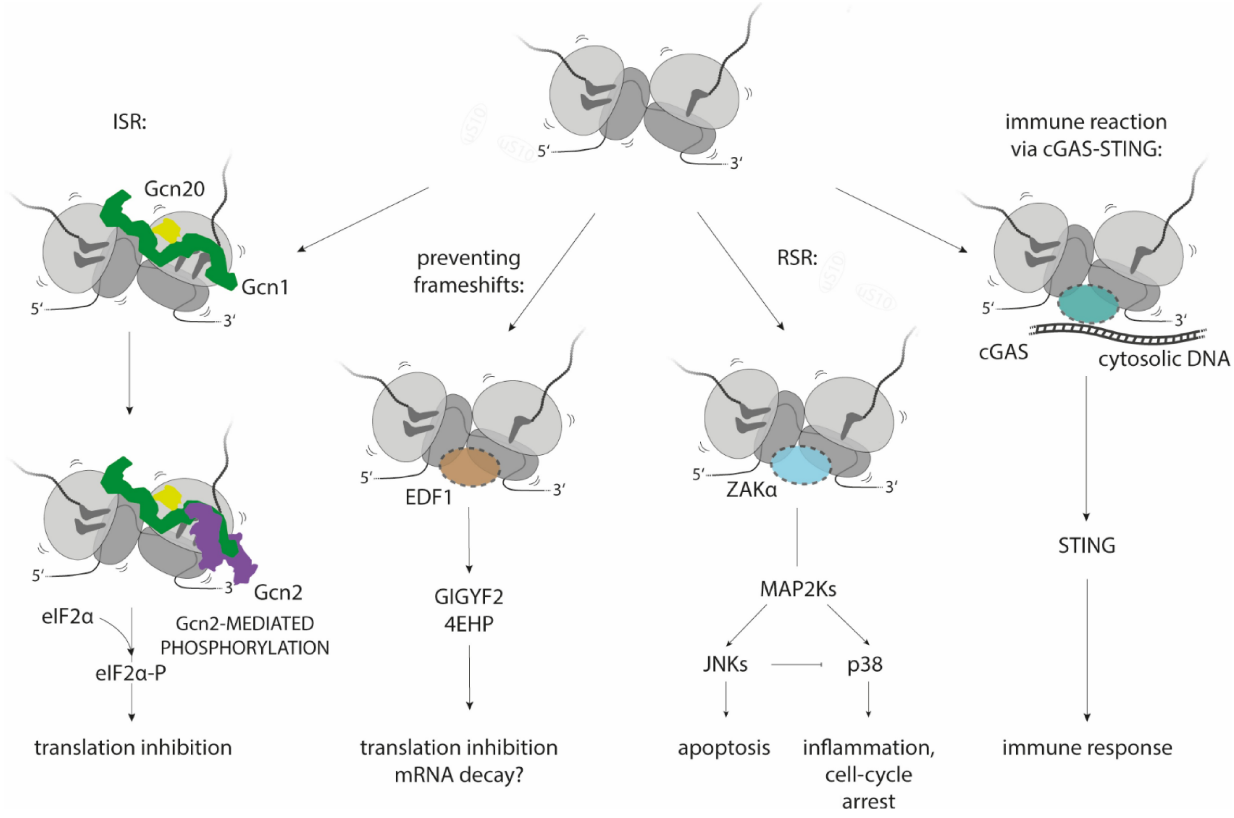

Figure 6. Other cellular responses to ribosome collisions. From left to right: The ISR kinase Gcn2 requires its co-activators Gcn20 and Gcn1 to phosphorylate eIF2α, leading to global translation inhibition. Binding of EDF1 to collided ribosomes downregulates translation initiation through GIGYF2 and 4EHP and possibly leads to mRNA decay. The ribotoxic stress response can be activated by collided ribosomes via ZAKα and downstream MAPK signalling pathways. In the presence of cytosolic DNA, collided ribosomes act as a co-activator of cGAS and thereby trigger immune responses via STING.

suppress frameshifting in collided ribosomes.[140] In fungi, this may be through competition for ribosome-binding with the fungal-specific elongation factor eEF3.[27,140,141] eEF3 in turn, is a driver of frameshifting in the absence of the important transcription factor Mbf1. As an additional function Mbf1 senses collisions and likely locks the head domain of the 40S subunit in position to prevent frameshifting.[27,140,142,143] Mbf1 is found bound to disomes together with Gcn1 in structural works.[27]

Similarly, in mammals, the ortholog of Mbf1, EDF1, acts on collided ribosomes by downregulating translation of mRNAs bearing collided ribosomes, together with a complex formed by GIGYF2 and 4EHP.[127,142,144,145]

Preventing additional ribosomes from engaging translation of problematic mRNAs which would ultimately end up as part of the collision themselves, prevents the RQC machinery from being overwhelmed. Reports are still conflicting on whether[145] or not[127] this activity is directly linked to mRNA degradation. In yeast, as discussed in 3.2, the two orthologs of GIGYF2, Smy2, and Syh1 (but no clear ortholog of 4EHP) have been identified. Both orthologs support NGD, but their recruitment differs from that of GIGYF2/4EHP as it does not seem to involve Mbf1.[125]

4.3. Activation of the ribotoxic stress response

The mammalian factor ZAKα, which was mentioned above as important for the activation of the ISR (see 4.1), has a second, better characterized role in activating the RSR. The term 'ribotoxic stress response' was introduced after the observation that various stresses that induce this response, such as different translation-inhibitors and toxins, acted on ribosomes. Many of them associate with or damage a region in the 28S rRNA, within the large ribosomal subunit.[146] Notably, ribosomes need to be actively translating to be targeted by the RSR.[146] The RSR, however, is not only activated by chemical stresses, but also by UV irradiation.[147] In the RSR, a mitogen-activated protein (MAP) kinase cascade is activated. A MAP3K (HCK, PKR, or ZAK) phosphorylates a MAP kinase kinase (MAP2K), which in turn phosphorylates MAP kinases, namely p38 and cJun N-terminal kinase (JNK), leading to further downstream responses. Those include the expression of pro-inflammatory cytokines, activation of the inflammasome,[148] metabolic regulation[149], as well as cell cycle arrest[150] and apoptotic cell death.[28,151] Recently, it has become clear that ribosome collisions,[28,149,152] as well as stalled ribosomes[149] can trigger the RSR via the longer splice variant of ZAK, ZAKα.[28,152] Ribosome collisions provide a stronger activation signal than single stalled ribosomes.[149] ZAKα contains two sensor domains in its C-terminus which bind ribosomes in part

redundantly, through the 18S rRNA of the small ribosomal subunit.[152] Upon ribosome binding ZAKα autophosphorylates,[28,152] which reduces its affinity to ribosomes and assists its cytoplasmic activity of activating MAP2K.[152] It is thought that the RSR gets activated when other response systems are overwhelmed.

4.4. Antiviral responses via cGAS-STING

An additional cellular response that is supported by ribosome collisions is an immune response via the cGAS-STING pathway.[153,154] Here, cGAS senses cytosolic (self or foreign) DNA and thereby contributes to innate immune responses to a large variety of pathogens. As a result, cGAS catalyses the formation of second messenger cyclic guanosine monophosphate-adenosine monophosphate (cGAMP) from ATP and GTP. cGAMP in turn binds to STING, activating expression of interferons and cytokines. It was recently shown that collided ribosomes can act as co-activators of cGAS, stimulating its DNA-dependent activity.[29] Here, collided ribosomes are bound directly by cGAS and act as a sign of translation stress during viral infection and possibly also in tumors. Upon deficiency of ASCC3, ZNF598, or RACK1 and hence an increased burden of collided ribosomes, interferon stimulated genes (ISGs) are expressed via the cGAS-STING pathway. However, collided ribosomes alone, without the presence of cytosolic DNA, are hardly able to activate cGAS.[29] These recent observations fit reports in which ASCC3 and ZNF598 negatively regulate antiviral ISG expression, and their depletion leads to an upregulation of ISGs and an antiviral state,[155,156] for example in case of poxvirus.

5. Responses to Ribosome Collisions in Bacteria

Ribosome collisions are a phenomenon that is not restricted to eukaryotes. Collisions also occur in bacteria, where a set of systems to counter ribosome collisions and other translation problems has equally evolved (Figure 7). Two recent reports[157,158] highlight the role of proteins that contain SMR-

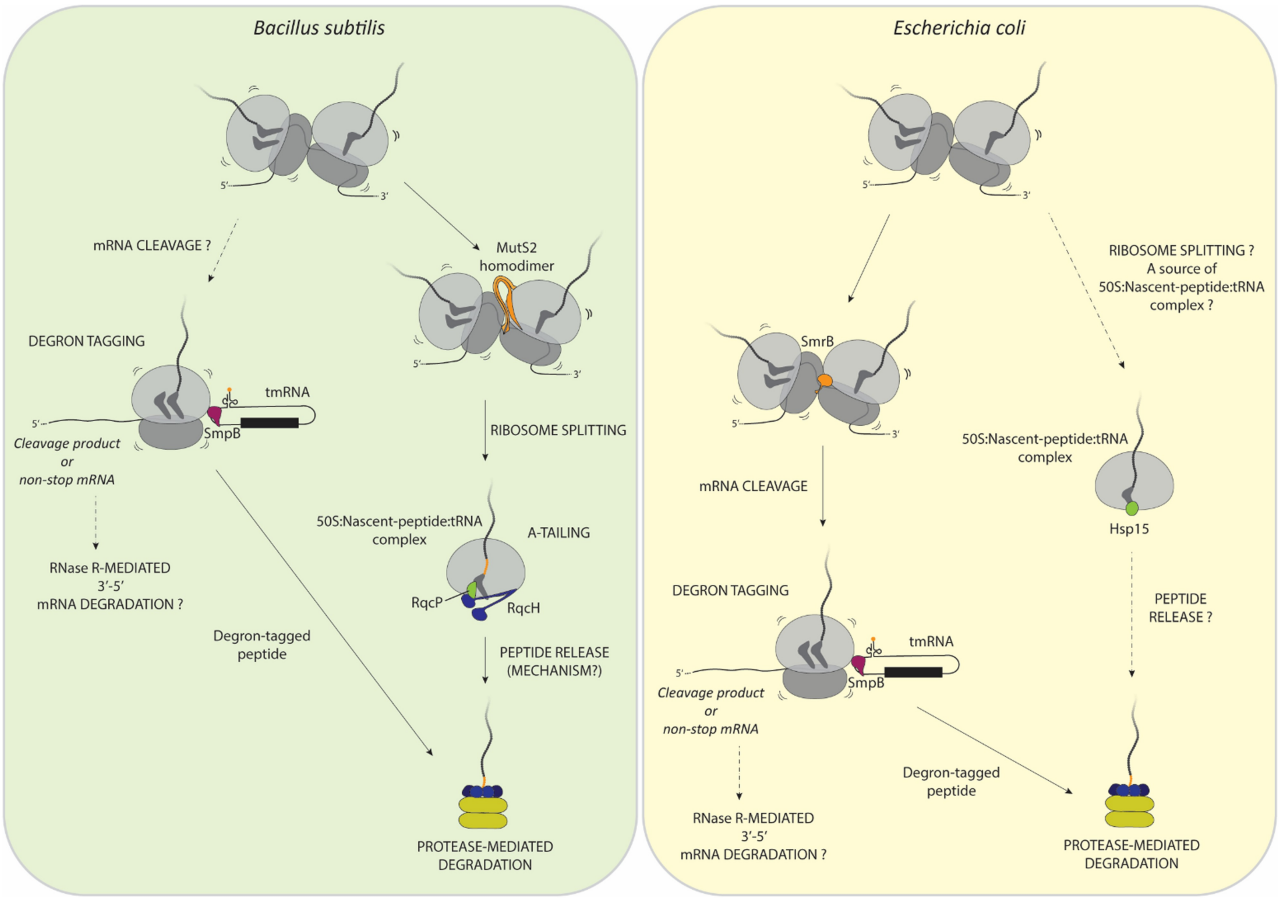

Figure 7. Overview of bacterial Ribosome-associated Quality Control pathways. (Left) In *B. subtilis*, dimeric MutS2 recognizes collided ribosomes and facilitates ribosome splitting. The resulting 50S:nascent peptide:tRNA complex is bound by RqcH and RqcP to add C-terminal alanine tails which function as a degron tag to target the nascent peptide for degradation. Ribosomes stalled at truncated or cleaved mRNAs are subjected to tmRNA-SmpB-mediated degron-tagging for degradation. An mRNA cleavage function has not been observed yet. As a protease, ClpXP is represented. (Right) In *E. coli*, ribosome collisions are recognized by SmrB which mediates mRNA cleavage. Ribosomes stalled on truncated/cleaved mRNAs are engaged by tmRNA-SmpB which adds a tmRNA-templated degron-tag to the nascent peptide to target it for protease-mediated degradation. As a protease, ClpXP is represented. Truncated or cleaved mRNAs are degraded in a 3′-to-5′ direction by RNase R. In case of dissociated 50S:nascent peptide:tRNA complexes, the peptidyl-tRNA is positioned in the P-site by Hsp15, which may be required as a prerequisite for peptide release. A ribosome dissociation function has not been observed yet.

domains in bacterial translation quality control. This domain is also present in yeast Cue2, the endonuclease that catalyzes cleavage of mRNA during NGD.[62]

5.1. Bacterial RQC in *Bacillus subtilis*

In *B. subtills*, collided ribosomes are subjected to quality control by SMR-domain containing protein MutS2.[157] MutS2 forms a homodimer and binds to the collided disome interface. Upon ATP-driven conformational changes, the ribosome is split and thus rescued. In the structural study, MutS2 was also hypothesized to be able to endonucleolytically cleave the mRNA in the collided disome, via the SMR-domain (which is not visible in the recently published structure). However, a very recent preprint suggests this is not the case.[159] Besides MutS2, the presence of an additional functionally redundant splitting factor in *B. subtilis* is hypothesized.[157] Splitting results in a peptidyl-tRNA:50S complex. This complex acts as a substrate for bacterial RQC by RqcH and RqcP, which bears great resemblance to eukaryotic CAT-tailing, although here, tails consist of alanine only. RqcH is the prokaryotic ortholog of yeast Rqc2, an essential player in eukaryotic CAT-tailing (see section 3.1) and catalyzes poly(alanine) tailing. The conserved residues D97 and R98 are required for binding of RqcH to tRNA.[160] RqcP binds to the peptidyl-tRNA:50S complex and is required for alanine tailing, but not by facilitating recruitment of RqcH. Instead, the factor was proposed to be required for translocation from an A/P hybrid tRNA state to a P-site state.[161] Alternatively, RqcP, which binds only to P-tRNA:50S complexes,[162] between the P- and E-site, was suggested to stabilize the peptidyl-tRNA in the P-site, thereby keeping the A-site free for incorporation of $tRNA^{Ala}$ by RqcH. This function is hypothesized to be equivalent to that of eIF5A[103] in yeast. In each incorporation cycle, RqcP must dissociate for transition of the P-site tRNA into the E-site.[163] Besides RqcP and RqcH, the reconstitution of alanine tailing *in vitro* requires tRNA, alanyl-tRNA synthetase, L-alanine, and ATP.[162] Alanine tails then act as a degron and allow for degradation by ClpXP or other proteases.[157,160]

5.2. Translation quality control in *Escherichia coli*

In *E. coli*, another SMR-protein, SmrB binds to collided disomes and cleaves mRNA upstream of the stalled ribosome.[158] The colliding ribosome is then left with an empty A-site and becomes a target of transfer-messenger RNA (tmRNA).[158] The tmRNA system represents the first translation quality control pathway that was discovered in bacteria. It generally deals with ribosomes stalled at the 3′-end of mRNAs that lack a stop codon. tmRNA displays features of both, tRNA and mRNA and is aminoacylated by alanyl-tRNA synthetase[164]. The mRNA-like part of tmRNA encodes an amino acid tag that acts as a degron and stimulates protease-mediated degradation when added to proteins expressed from non-stop/truncated mRNAs.[165] At the very 3′-end of such RNAs, the ribosomal A-site is not occupied by an mRNA or tRNA. tmRNA, in complex with the protein SmpB is then accommodated in this position. Upon peptidyl transfer, when the tmRNA alanine is added to the peptide chain, the tmRNA takes over the role of mRNA template and is then decoded. Thereby, the last amino acids of the degron are added, allowing for degradation of the nascent peptide via ClpXP and other proteases.[166,167] This process is called trans-translation. Non-stop mRNAs are then degraded by the 3′-to-5′-exonuclease RNase R, in a process that depends on both tmRNA and SmpB.[168] Though only described in *E. coli*, it is likely that RNase R has the same role in *B. subtilis* as well, as tmRNA, SmpB and RNase R are transcribed from the same cluster in that organism.[169] Although trans-translation does not require ribosomal collisions, tmRNA has been shown to at least be involved in rescuing collided ribosomes after endonucleolytic cleavage between the collided ribosomes by SmrB in *E. coli.* As discussed in 5.1, an endonucleolytic activity has not been observed in *B. subtilis* (yet?). However, a similar mechanism would be plausible should such an activity be found. An ortholog of RqcP also exists in *E. coli*: Hsp15. Hsp15 binds to 50S subunits formed by dissociated polysomes under conditions of heat shock and chloramphenicol.[170] Hsp15 requires P-tRNA:50S for binding[170] and was suggested to translocate P-tRNA from A/P site to P-site.[171] Although the significance of this is unknown, it was hypothesized to be required for aiding in the P-tRNA release. To act on P-tRNA:50S complexes resulting from collided ribosomes, a splitting activity would likely be needed, which has not been described yet.

5.3. Alternative rescue factors

In the absence of the tmRNA system, which usually constitutes the main rescue pathway[161] or when that system is overwhelmed, ribosomes stalled at the 3′-end can also be rescued by rescue factors ArfA (for alternative rescue factor A in *E. coli*) or BrfA (in *B. subtilis*). These factors recruit the canonical release factor 2 (RF2) that cleaves the peptidyl-tRNA from 70S ribosomes.[172–175] In this case, contrary to trans-translation in the tmRNA system, the resulting proteins are not degraded. Another alternative rescue factor, ArfB (in *E. coli*) can directly cleave peptidyl-tRNA.[176,177] These rescue factors have not been linked to ribosome collisions, yet. However, an involvement in resolving ribosome collisions, e.g., as a backup mechanism for trans-translation following mRNA cleavage, would be plausible.

6. Conclusion and Outlook

Much progress has been made recently to better understand pathways that respond to translation stress. Major advances include the discovery of (1) ribosome collisions as a recognition platform for the various translation stress sensors[178] (2) ubiquitin as an important mark to signal translation stress in eukaryotes[120] and (3) highly conserved translation quality control pathways from bacteria to eukaryotes.[93]

It is now becoming increasingly clear that cells mount a multi-pronged response upon ribosome collisions to counteract

translation stress at the level of the collision itself, of translation of affected mRNAs, as well as general translation and on the cellular and organismal levels. To maintain or re-establish cellular homeostasis, it is important that those responses are carefully balanced within the cell, be it in terms of balance between different responses, or in terms of carefully tuning each response itself. Fine-tuning has been observed, e.g., according to the type of damage[150] and the amount of collisions.[26,28] Furthermore, splitting, re-use and degradation of ribosomes, as well as mRNA degradation, compared to ribosome splitting and resuming translation on the still-intact mRNA, need to be balanced as well.

In conclusion, future studies are needed to understand both, the different response pathways, and their interplay.

As mentioned at the beginning of this review, pathways that deal with ribosome collisions have been linked to various (neurodegenerative) diseases. One of the goals of all efforts to understand the rescue pathways dealing with ribosome collisions will thus be to study the influence of ribosome collisions and those pathways on pathogenesis. Another aim will be to inform and enable future attempts towards improved diagnostic and therapeutic tools.

At this point, it only remains to be noted that not all translation problems lead to ribosome collisions and other rescue pathways exist, e.g., reacting specifically to non-collided, stalled ribosomes.[179]

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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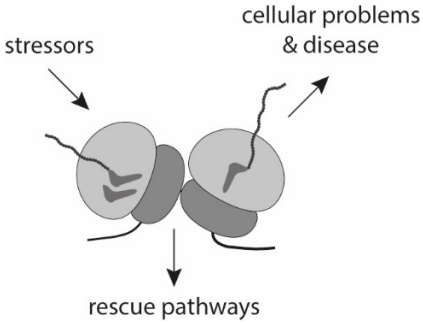
REVIEW

The discovery of ribosome collisions as the recognition platform of translation problems caused by various factors represents a cornerstone in the field of translation surveillance. We summarize our current knowledge about the pathways that target collided ribosomes. These pathways do so directly - by removing or recycling ribosomes, peptides, and mRNA - or indirectly - by modulating translation and cellular fate.



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1 – 15

Molecular Highway Patrol for Ribosome Collisions