Review

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Ribosome recycling in mRNA translation, quality control, and homeostasis

<https://doi.org/10.1515/hsz-2019-0279>

Received June 4, 2019; accepted October 22, 2019; previously published online October 29, 2019

Abstract: Protein biosynthesis is a conserved process, essential for life. Ongoing research for four decades has revealed the structural basis and mechanistic details of most protein biosynthesis steps. Numerous pathways and their regulation have recently been added to the translation system describing protein quality control and messenger ribonucleic acid (mRNA) surveillance, ribosome-associated protein folding and post-translational modification as well as human disorders associated with mRNA and ribosome homeostasis. Thus, translation constitutes a key regulatory process placing the ribosome as a central hub at the crossover of numerous cellular pathways. Here, we describe the role of ribosome recycling by ATP-binding cassette sub-family E member 1 (ABCE1) as a crucial regulatory step controlling the biogenesis of functional proteins and the degradation of aberrant nascent chains in quality control processes.

Keywords: ABC proteins; molecular machines; mRNA surveillance; ribosome recycling; ribosomopathies; specialized ribosomes; translational control.

Introduction

The origin of translation is inextricably related to the universal genetic code. Evolution has assembled large ribonucleoprotein (RNP) complexes to catalyze translation and developed a sophisticated network to regulate and survey it. Genetic information is stored in large deoxyribonucleic acid (DNA) molecules termed chromosomes. Highly regulated cellular mechanisms allow the transcription of a

requested gene into messenger ribonucleic acid (mRNA). Decoding of the mRNA triplet code into a polypeptide chain is coordinated and catalyzed within the ribosome, a macromolecular factory comprising ribosomal RNA (rRNA) and proteins (rps) that form the translation machinery (70S ribosomes in Bacteria and Archaea; 80S ribosomes in Eukarya) out of a small (30S/40S) and a large (50S/60S) subunit. Transfer RNAs (tRNAs) act as adapter units, carrying an antisense triplet to decode the mRNA, and deliver the correct amino acid to elongate the polypeptide chain accordingly. The tightly coupled assembly, translocation, and disassembly of the ribosome as well as the delivery of tRNA are managed by translation factors and divided into four phases: initiation, elongation, termination, and ribosome recycling (Hellen, 2018; Shirokikh and Preiss, 2018; Stein and Frydman, 2019). However, the biogenesis of functional proteins requires precise reconciliation of translation with protein folding, chemical modification, and eventually membrane insertion or translocation. Thus, these processes are likewise coordinated by ribosome-associated factors (Kramer et al., 2019; Waudby et al., 2019). Ribosome heterogeneity is debated to alter key functions of single ribosomes (Ferretti and Karbstein, 2019). Additionally, quality control pathways, partly triggered at macromolecular assemblies of multiple ribosomes, dynamically regulate mRNA and protein homeostasis (Joazeiro, 2019). By fine-tuning the translation machinery, cells can switch between global operation modes such as survival, proliferation, differentiation, and apoptosis (Holcik and Sonenberg, 2005; Chang and Stanford, 2008). Imbalances in this sensitive system can therefore lead to various diseases, including neurodegeneration, cancer, or inherited ribosomopathies, for reasons we are just beginning to understand (Gao et al., 2017; Robichaud et al., 2018; Tahmasebi et al., 2018). Here, we discuss the structural and regulatory aspects of translation with an emphasis on ribosome recycling, a crucial step at the intersection of numerous translational pathways.

Features of the ribosome

Ribosomal subunits consist of universally conserved core rRNA and rps but also of peripheral elements specific for

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each phyla, organism, and even organelle (Bieri et al., 2018; Rodnina, 2018; Jobe et al., 2019). Assembled ribosomes are highly dynamic and undergo large conformational rearrangements both among the two subunits against each other as well as within each subunit (Prabhakar et al., 2017; Rodnina, 2018; Jobe et al., 2019). The interface composes three ribosomal tRNA binding sites for aminoacylated tRNA (A-site), peptidyl-tRNA (P-site), and deacylated tRNA (E-site, exit site) (Rheinberger et al., 1981; Agrawal et al., 1996; Stark et al., 1997). The conserved helix 44 (h44) of the 16S/18S rRNA maintains subunit association during translocation and contributes to translation fidelity (Jenner et al., 2010; Qin et al., 2012; Liu and Fredrick, 2016). Catalysis takes place on the large ribosomal subunit, in the peptidyl transferase center (PTC) constituted by the 23S/28S rRNA component. The emerging polypeptide moves through the exit tunnel, which is ~10 nm long and allows folding of the nascent chain into secondary structures in a shielded environment (Nilsson et al., 2015; Komar, 2018). On the solvent-exposed side of the large subunit, the N terminus of the partially folded polypeptide encounters various regulatory and processing factors like folding chaperones, signal recognition particles for trafficking, and enzymes for co-translational modification (Kramer et al., 2019). The sarcin-ricin loop (SRL) in the 23S/28S rRNA and the P-stalk control translational factors, which orchestrate each phase of protein synthesis (Voorhees and Ramakrishnan, 2013).

Ribosome synthesis requires over 200 additional proteins and is one of the most energy-consuming processes in the cell (Kressler et al., 2017; Pena et al., 2017). Disorders in ribosome biogenesis are connected to various human diseases referred to as ribosomopathies (Narla and Ebert, 2010; Mills and Green, 2017; Tahmasebi et al., 2018). On the other hand, deactivation of ribosomes immediately arrests the synthesis of all proteins and can protect cells from viral assault, starvation, and endoplasmic reticulum (ER) stress by unfolded proteins. In these critical situations, cells monitor definite ribosome loss and cleave rRNA by RNase L in innate immunity, RNase PH during nutrient shortage, and inositol requiring enzyme-1 β (IRE1 β) in the context of the unfolded protein response (UPR) (Wreschner et al., 1981; Iwawaki et al., 2001; Basturea et al., 2011). Thus, ribosome homeostasis is critical during cell stress, development, and proliferation (Mills and Green, 2017; de la Cruz et al., 2018).

Translation termination

Canonical termination involves recognition of the stop codon in the ribosomal A-site and peptide release by the cooperative action of class I and class II release factors

(RFs). Termination in Bacteria differs significantly from the eukaryotic mechanism, while Archaea follow the eukaryotic route, albeit with a fractional set of factors. Eukaryotic/archaeal (e/a) class I e/aRF1 comprises three flexible domains and structurally mimics a tRNA molecule (Song et al., 2000). e/aRF1 is delivered to the ribosomal A-site in a stable ternary complex with the translational GTPases eRF3 (Zhouravleva et al., 1995; Alkalaeva et al., 2006) or aEF1 α (Kobayashi et al., 2012). In Eukarya, guanosine triphosphate (GTP) hydrolysis and dissociation of eRF3 allows the full accommodation of eRF1 in the A-site, leading to peptide release (Figure 1). The decisive fidelity and efficiency of decoding factor accommodation during elongation (aa-tRNA), termination (eRF1), and mRNA surveillance (ePelota) depends on a complex interplay between (i) decoding and (de-)stabilization of mRNA in the A-site, (ii) conformational changes of the small ribosomal subunit, in bacteria also referred to as domain closure (Ogle et al., 2002), (iii) activation of the respective delivery GTPase, and (iv) structural rearrangements within the decoding factor (Alkalaeva et al., 2006; Shao et al., 2016). The conserved NIKS motif in the N-terminal (N) domain of eRF1 recognizes the stop codon in a precise geometric arrangement, which includes mRNA compaction and stacking interaction with the A¹⁸²⁵ base (rabbit) in the 40S ribosomal h44. The additional conserved GTS and YxCxxxF motifs in eRF1 discriminate against sense codons (Brown et al., 2015). Upon successful decoding, GTP hydrolysis within eRF3 is activated *via* the SRL, and eRF3·guanosine diphosphate (GDP) dissociates. Thereby, the middle (M) domain of eRF1 is liberated and swings into the PTC, where the universally conserved GGQ motif assists the nucleophilic attack of a water molecule to release the nascent chain (Frolova et al., 1999; Brown et al., 2015). Notably, these structural rearrangements in class I RFs, rather than peptide release, mark the formation of a post-termination complex (post-TC) ready for ribosome recycling.

Ribosome recycling

Within the canonical translation cycle, terminated ribosomes are recycled into free subunits. Ribosome recycling is tightly regulated and represents a key process in translational control. In Eukarya and Archaea, ribosome recycling is orchestrated by the essential and conserved ATP-binding cassette sub-family E member 1 (ABCE1) in collaboration with the class I RFs in the A-site of the post-TC (Pisarev et al., 2010; Barthelme et al., 2011; Shoemaker and Green, 2011). ABCE1 is a non-membrane-associated

Figure 1: Molecular mechanism of translation termination and ribosome recycling.

(A) The pre-termination complex (PDB 5LZT) comprises 80S ribosomes, mRNA, deacylated E-site tRNA, peptidyl-tRNA in the P-site, eRF1 in the A-site, and eRF3-GTP at the GTPase center. The conserved NIKS and GTS motifs in the N-terminal domain (NTD) of eRF1 recognize the stop-codon in the decoding center. The middle-domain (MD) with the GGQ motif is locked by eRF3-GTP, thus the nascent chain in the PTC has not yet been released. (B) After GTP hydrolysis and eRF3 dissociation, ABCE1 occupies the GTPase center forming the 80S pre-splitting complex (PDB 5LZV). The MD of eRF1 is in the post-termination state, swung out into the PTC (black arrow), where a water molecule coordinated by the GGQ motif can release the nascent chain. The N-terminal FeS domain (FeSD) of ABCE1 is associated with the C-terminal domain (CTD) of eRF1. The control site II is in a semi-closed state, with NBD1 and NBD2 slightly approaching each other while site I remains open. (C) Occlusion of two ATP molecules by ABCE1 splits the ribosome apart by a 150° rotation of the FeSD (black arrow) toward helix 44 and ABCE1 remains bound at the 40S subunit, reconstituting the post-splitting complex.

ABC-type ATPase (70 kDa) and represents the only member of ABC subfamily E. This ribosome recycling factor is evolutionarily conserved and essential in all organisms except Bacteria. Like in a typical ABC protein, the two nucleotide-binding domains (NBDs) of ABCE1 are arranged head-to-tail and harbor two composite nucleotide-binding sites at their interface (Karcher et al., 2005, 2008; Barthelme et al., 2011). ATP binding in both sites leads to tight dimerization (closure) of the NBDs, while subsequent ATP hydrolysis and release of inorganic phosphate allow their disengagement. Thus, the NBDs perform a tweezer-like motion, which is mechanically coupled to associated domains to either transport substrates across the membrane or remodel nucleoprotein complexes (Hopfner, 2016; Thomas and Tampé, 2018). Additionally,

ABCE1 possesses a unique N-terminal iron-sulfur (FeS) cluster domain, which harbors two diamagnetic [4Fe-4S]²⁺ clusters, a helix-loop-helix insertion in NBD1, and a small and flexible hinge region, which connects the two NBDs and supports their orientation (Karcher et al., 2005; Barthelme et al., 2007, 2011). Mutations in conserved functional motifs of ABCE1 are lethal at early embryonic stage and have asymmetric effects on the overall ATPase activity of the protein (Andersen and Leever, 2007; Barthelme et al., 2011; Nürenberg-Goloub et al., 2018). Similar to translational GTPases, ABCE1 engages with the ribosomal P-stalk of 70S/80S ribosomes (Imai et al., 2018) before it attaches near the GTPase activating center and contacts the C-terminal (C) domain of the respective class I RF thereby forming the pre-splitting complex (Figure 1)

(Becker et al., 2012; Shao et al., 2016). Interestingly, ABCE1 promotes eRF1-mediated release of short peptides in the absence of eRF3, though with lower efficiency, suggesting a yet uncharacterized function in translation termination (Shoemaker and Green, 2011). The two structurally and functionally distinct sites allow ABCE1 to distinguish splitting-competent ribosomes (Nürenberg-Goloub et al., 2018). More precisely, a control site with an intrinsically low ATP-turnover rate holds ABCE1 at the pre-splitting complex while the other site probes for a conformation, which would induce ribosome splitting. If ABCE1 fails to achieve this conformation, it can be released from the pre-splitting complex and bind another ribosome (Nürenberg-Goloub et al., 2018; Gouridis et al., 2019). In case the complex comprises a splitting-competent ribosome, ATP-dependent conformational changes of the NBDs and the FeS cluster domain induce a translocation-like ribosome destabilization (Pisarev et al., 2010). Finally, ATP occlusion and closure of both sites displaces the FeS domain, which protrudes between the ribosomal subunits causing their dissociation (Heuer et al., 2017; Nürenberg-Goloub et al., 2018). After splitting, ABCE1 remains at the small ribosomal subunit, establishing the post-splitting complex (post-SC, Figure 1) (Barthelme et al., 2011; Kiosze-Becker et al., 2016; Heuer et al., 2017). ATP hydrolysis in both sites of ABCE1 is downregulated within the post-SC (Nürenberg-Goloub et al., 2018), where it prevents rejoining of the large ribosomal subunit (Heuer et al., 2017). Deacylated tRNA and mRNA are removed from separated 40S subunits by the translation initiation factors (IFs) eIF1, eIF1A, and eIF3 (Pisarev et al., 2010), ligatin, or multiple copies in T-cell lymphoma-1/density regulated protein (Skabkin et al., 2010) in an ABCE1-independent manner *in vitro*. The additional presence of eIF2 and initiator Met-tRNA^{Met} promotes re-initiation of translation at up- and downstream start-codons on the same mRNA prior to its ejection and the addition of eIF4F ensures 3'-directionality of re-initiation (Skabkin et al., 2013).

Within the post-SC, ABCE1 can link ribosome recycling to translation initiation (Heuer et al., 2017; Mancera-Martinez et al., 2017; Gerovac and Tampé, 2019) and has been proposed to act in 5'-cap and poly-A independent mRNA circularization (Afonina and Shirokov, 2018). First indications on the role of ABCE1 in initiation was gained from human, fruit fly, and yeast before its definite assignment to ribosome recycling (Dong et al., 2004; Yarinin et al., 2005; Chen et al., 2006; Andersen and Leever, 2007). Notably, the non-essential initiation factor eIF3j (Hcr1 in yeast) assists ABCE1 during recycling of terminated 80S ribosomes at stop codons *in vivo* (Young and Guydosh, 2019). In cooperation with other

IFs, Hcr1 controls stringent start codon selection during translation initiation and assists eRF3 ejection from 80S ribosomes after translation termination (Elantak et al., 2010; Beznoskova et al., 2013). Both ABCE1 and Hcr1 are associated within the multifactor complex free of ribosomes, rising the assumption of a pre-formed unit functional in termination, ribosome recycling, and (re-) initiation (Asano et al., 2000; Dong et al., 2004). Therein, ABCE1 not only restocks the cellular pool of free ribosomal subunits, but schedules the selective recycling of post-TCs and gates small ribosomal subunits toward translation initiation (Heuer et al., 2017; Nürenberg-Goloub et al., 2018).

Apart from its multiple essential roles related to ribosome recycling, ABCE1 has vaguely defined functions as RNase L inhibitor (Bisbal et al., 1995) and in the assembly of numerous virions including those of human immunodeficiency virus (Dooher et al., 2007; Anderson et al., 2019). Thus, the molecular mechanism of this versatile protein is of high interest in diverse fields of research, including therapeutics against human diseases like rabies or cancer (Huang et al., 2010; Lingappa et al., 2013).

mRNA surveillance and ribosome-based quality control

Elongation and termination can fail for numerous reasons, resulting in stalled ribosomes occupied by faulty mRNA and non-functional, potentially harmful polypeptides. The cellular quality control machinery aims to eliminate mRNA, polypeptides, and/or damaged ribosomes at the earliest time point, while they are still unambiguously connected to each other. Thus, quality control directly takes place at the ribosome, which allows the cell to simultaneously target aberrant polypeptides to the proteasome, degrade the respective mRNA, and activate stress response signaling (Brandman and Hegde, 2016; Joazeiro, 2019). Bacteria utilize three different systems to resolve the stalled ribosomal complex and eventually target mRNA and the nascent chain for degradation. *Trans*-translation of the trapped mRNA (tmRNA) molecule appends a specific sequence to the aberrant peptide that serves as a degradation signal. Notably, bacteria also employ tmRNA to monitor co-translational processes (Hayes and Keiler, 2010) and during starvation to quickly adapt to environmental conditions (Keiler, 2008). Alternative rescue factors (ArfA and ArfB) promote translation termination, peptide release, and ribosome recycling without tagging the nascent chain (Keiler et al.,

1996; Keiler, 2015; Huter et al., 2017). Finally, the bacterial ribosome-based quality control (RQC) factor RqcH recognizes peptidyl-tRNA bound to 50S ribosomal subunits and induces nascent chain degradation by appending a poly-alanine tail, suggesting the presence of another yet elusive recycling pathway for stalled ribosomes (Lytvynenko et al., 2019). The three bacterial quality control systems are essential or redundant in some bacterial species but their high evolutionary distribution points toward a substantial contribution to cellular fitness during environmental stress (Keiler, 2015; Lytvynenko et al., 2019).

The eukaryotic mRNA surveillance and RQC system comprises multistep response pathways for a variety of translational errors induced by (i) truncated or highly structured mRNAs (no-go decay, NGD), (ii) open reading frames (ORFs) either lacking (no-stop decay, NSD) or containing a (premature) in-frame stop codon (nonsense-mediated decay, NMD), (iii) an aa-tRNA shortage, (iv) polypeptides blocking the ribosomal exit tunnel, or (v) defective ribosomes (18S-NRD) (Brandman and Hegde, 2016; Buskirk and Green, 2017; Joazeiro, 2019). Notably, the NMD pathway is tightly coupled to translation termination and employs the canonical eRF1-eRF3 complex, which interacts with up-frameshift (UPF) translational regulators to induce mRNA and protein degradation (Karousis and Muhlemann, 2019; Kurosaki et al., 2019). In contrast, the hallmark of all other quality control pathways is the stalled 80S ribosome, with either no mRNA or a sense codon in the decoding site. Stalled 80S ribosomes recruit the stop codon-independent class I RF ePelota (Dom34 in yeast), delivered by GTPase Hbs1 (Chen et al., 2010; Becker et al., 2011). Hbs1 senses the lengths of 3'-mRNA overhangs at the 80S (Shoemaker and Green, 2011) and recruits the superkiller (SKI) complex involved in fast degradation of the aberrant mRNA, in concert with the exosome and other nucleases (Saito et al., 2013; Joazeiro, 2019). Subsequently, Hbs1 dissociates, leaving a post-TC with intact peptidyl-tRNA in the P-site and ePelota in the A-site. This complex is a substrate for ABCE1 (Pisarev et al., 2010; Shoemaker and Green, 2011; Becker et al., 2012; Nürenberg and Tampé, 2013). The dynamic regulation of ribosome abundance by ePelota and ABCE1 plays a crucial role during erythropoiesis (Mills et al., 2016; Khajuria et al., 2018). Erroneous peptide targeting and stress signaling take place downstream of recycling at 60S subunits, harboring the intact peptidyl-tRNA and involve numerous factors with yet incompletely defined roles and operation modes, including a homolog of the bacterial RqcH (Brandman and Hegde, 2016; Joazeiro, 2019). Especially, the NMD pathway is tightly coupled to

post-transcriptional mRNA processing events, mRNA and protein homeostasis, and deterministic regulation of gene expression (He et al., 2003; Yap and Makeyev, 2013). Thus, it is not surprising that failure of NMD leads to various human diseases (Jaffrey and Wilkinson, 2018; Kurosaki et al., 2019).

Information about archaeal mRNA surveillance and RQC pathways is scarce, but appears to include eukaryotic features: the stop codon-independent RF aPelota is delivered to stalled ribosomes by aEF1 α (Kobayashi et al., 2010) and recycling of stalled post-TCs likewise depends on ABCE1 (Becker et al., 2012). Archaea possess a yet uncharacterized RqcH homolog (Lytvynenko et al., 2019). It remains largely elusive how Archaea deal with translation and transcription errors, but a simplified eukaryotic system appears to be most likely. Furthermore, it remains to be clarified how archaeal co-translational quality control pathways are involved in global cellular regulation.

Supramolecular ribosome assemblies

Supramolecular assemblies reaching from di-ribosomes to various topologies of polyribosomes can alter translation activity. Formation of defined di-ribosomes by collisions depend on the availability of translation and ribosome recycling factors as well as the sequence of the mRNA. Such collided di-ribosomes are subject to NGD and/or RQC (Simms et al., 2017; Juskiewicz et al., 2018; Ikeuchi et al., 2019). Polysomes dynamically reorganize during progressive ribosome loading onto an mRNA molecule, establishing contacts between conserved sites on the ribosomal surface (Afonina and Shirokov, 2018; Gohara and Yap, 2018). As inefficient late-stage polysomes were discussed to be collided ribosomes, their formation possibly depends on the availability of translation factors. Thus, ribosome recycling is likewise involved in translation regulation by ribosome assemblies.

Defined eukaryotic di-ribosomes (Figure 2) are formed in the context of RQC and mRNA surveillance pathways (Simms et al., 2017; Juskiewicz et al., 2018; Ikeuchi et al., 2019). If the rate of translation initiation on an mRNA exceeds its elongation, termination, and/or ribosome recycling capacity, ribosomes likely collide within or at the end of the ORF, respectively. Collided ribosomes have a conserved dimerization interface, which is recognized by the E3 ubiquitin ligases ZNF598/Hel2 in rabbit/yeast. The minimal recognition motif is a di-ribosome. Upon ZNF598

Figure 2: Supramolecular ribosome assemblies.

(A) Collided yeast and mammalian di-ribosomes establish a specific interface, mainly involving 40S subunits (PDB 6I70). This interface is recognized by E3 ubiquitin ligases Hel2 (ZNF598 in mammals) and Not4, which specifically ubiquitinate ribosomal proteins uS3, uS10, and eS7, thereby initiating NGD and RQC. The ribosomal protein Asc1 (RACK1 in mammals) is not ubiquitinated but essential for interface recognition. (B) In helical polysomes (PDB 4V3P), 40S subunits (white) and mRNA are sequestered inside the helix, while the 60S subunits (blue) and nascent chains are exposed to the cytosol. The ribosomal P-stalks (red) are accessible to translation factors. Individual disome units in the helix resemble the stalled di-ribosome in panel (A), with a similar orientation and interface between individual ribosomes. Thus, specific ribosome ubiquitination might explain the reduced translation activity of helical polysomes.

binding, the stalled and collided ribosomes are sequentially ubiquitinated at ribosomal proteins uS10, eS10, and uS3. Elongation is arrested by a yet unknown mechanism. Ribosomes are recycled by ePelota and ABCE1, and aberrant nascent chains and mRNA are degraded *via* NGD and RQC in an NGD^{RQC+} response (Simms et al., 2017; Sundaramoorthy et al., 2017; Juszkiwicz et al., 2018; Ikeuchi et al., 2019). Interestingly, a distinct ubiquitination pathway of ribosomal protein eS7 by the E3 ligase Not4 induces an NGD^{RQC-} response, which does not feature degradation of the nascent chain (Ikeuchi et al., 2019). Strikingly, Not4 also mediates ABCE1 ubiquitination in ribosome-associated control of mitophagy (Wu et al., 2018), illustrating the intricate interplay of ribosome recycling and quality control in decisive cellular events. Thus, higher-order ribosome architecture can induce multiple quality control pathways, explaining how cells may distinguish between intended ribosome pausing and situations necessitating intervention to prevent synthesis of aberrant products. As an interesting consequence, the cells' definition of 'aberrant' mRNA and nascent chains becomes dependent on the cellular context, e.g. proliferation or differentiation status and environmental conditions (Juszkiwicz et al., 2018).

In general, higher-order ribosome arrangement modulates protein biosynthesis rates and mRNA stability and enables spatial and temporal control over translation as shown for neurons (Graber et al., 2013; Sossin and Costa-Mattioli, 2019). Interaction of poly-A binding proteins at the 3'-end and the eIF4 cap-binding complex at the 5'-end of juvenile cytosolic mRNA induce a pseudo-circular architecture, which is a widely accepted model (Hinnebusch, 2017). During initial rounds of translation, the spatial proximity of 3'- and 5'-ends allows rapid initiation of recycled ribosomes on the same mRNA. Thus, translation is most efficient within this initial phase, and polysomes have a ring-type shape corresponding to circular mRNA (Kopeina et al., 2008; Behrmann et al., 2015). Notably, approximately 5% of human 80S ribosomes in the actively translating polysome fraction are pre-splitting complexes resembling eRF1 in the post-termination state and ABCE1 (Behrmann et al., 2015). Strikingly, ring-type polysomes were equally observed on synthetic mRNAs that lacked a poly-A tail or 5'-cap, suggesting a distinct or more general mechanism of mRNA circularization (Afonina et al., 2015). As translation initiation occurs at the 5'-end of the mRNA and ribosome recycling follows termination at the 3'-end of the ORF, the

multifactor complex including ABCE1 may spatially and temporarily connect these fundamental processes at the post-SC (Heuer et al., 2017; Gerovac and Tampé, 2019).

With progressive ribosome load, eukaryotic polysomes acquire a three-dimensional (3D) helical structure (Figure 2), and the translation efficiency constantly drops (Kopeina et al., 2008; Brandt et al., 2010; Afonina et al., 2015). Interactions between the ribosomes involve 40S and 60S ribosomal subunits, including the ribosomal P-stalk, and rRNA expansion segments (ES) (Brandt et al., 2010; Myasnikov et al., 2014). The mRNA is shielded from the cytosol, and the peptide-exit sites are exposed. Interestingly, the previously enigmatic function of the rRNA ES was suggested to stabilize 3D polysomes. However, rRNA ES are also crucial for recruitment of the N-terminal acyl-transferase NatA (Knorr et al., 2019) and possibly other nascent chain processing factors. It has been speculated that the severe decrease of translation efficiency in 3D helical polysomes originates from ZNF598-mediated ribosome arrest (Juszkiewicz et al., 2018) (Figure 2). Therefore,

it remains a challenging objective to investigate the mechanism of translational arrest and resumption versus quality control within 3D helical polysomes in light of the diverse *in vivo* situations.

Translation regulation

Transcription and translation determine the protein composition and thereby define the function and fate of a cell. The levels of mRNA and protein only correlate with approximately half of eukaryotic genes (Vogel and Marcotte, 2012). The reason for this discrepancy lies within the dynamic interplay of regulatory mechanisms involving the mRNA, additional factors, and the ribosome itself (Figure 3). We would like to introduce the basics of eukaryotic translation regulation using examples of recent structural insights and discuss the impact of ribosome recycling and ribosome heterogeneity on the biosynthesis of functional proteins.

Figure 3: Translational control.

(A) Translation of the pseudo-circular eukaryotic mRNA is regulated by *cis* (orange) and *trans* (blue) factors and largely depends on the ribosome-recycling activity of ABCE1. Translation is initiated at the small ribosomal subunit, which can be delivered by ABCE1 within the post-splitting complex. A pre-initiation complex scans the mRNA in 5' to 3' direction to find a start codon, where initiation is accomplished and elongation begins. Ribosome recycling by ABCE1 promotes re-initiation on the main ORF start codon after translation of an uORF. Terminated, stalled, unrecycled, and hibernating 80S ribosomes are split by ABCE1 before individual subunits can re-enter the translation cycle. (B) The CrPV IRES (PDB 6D9J) mimics parts of the E-site tRNA and mRNA to promote eEF2-mediated ribosome translocation and to initiate translation of viral proteins, independent of the cellular translation initiation machinery. (C) IFRD2 prevents translation in hibernating ribosomes by blocking the P-site and the mRNA channel. Z-site tRNA has recently been visualized in hibernating ribosomes in the presence and absence of IFRD2 (PDB 6MTC). Its detailed role remains to be elucidated.

Eukaryotic mRNA translation is in general controlled by *cis* and *trans* regulators (Kozak, 2005; Sonenberg and Hinnebusch, 2009; Hershey et al., 2012; Leppek et al., 2018). *Trans* regulators include all translation factors, mRNA-binding proteins but also functional cellular RNAs such as micro- and long non-coding RNAs. Examples of *trans* factors inhibiting translation are interferon-related developmental regulator 2 (IFRD2) and Z-site tRNA specifically bound in proximity of the ribosomal E-site (Brown et al., 2018) (Figure 3). *Cis* regulators are secondary structures or specific sequences on the mRNA itself, e.g. stem loops, internal ribosomal entry sites (IRES), and upstream ORFs (uORFs). Thereof, a cryo-electron microscopy (cryo-EM) study demonstrated that ribosome hijacking by cricket paralysis virus (CrPV) IRES involves structural mimicry of E-site tRNA (Figure 3) (Muhs et al., 2015). Strikingly, epigenetic imprinting of mRNA during transcription adds another level to translational *cis*-regulation (Slobodin et al., 2017). uORFs have versatile effects on eukaryotic gene expression relying on *cis*- and *trans*-regulatory elements (Wethmar, 2014; Weisser et al., 2017), and are readily exploited in cancer cells (Sriram et al., 2018). Ribosome recycling by ABCE1 precedes binding of (re-)initiation factors to the intersubunit side of 40S ribosomes after translation termination on uORFs (Skabkin et al., 2010, 2013; Lomakin et al., 2017). In the absence of ABCE1, 80S post-TCs can re-initiate on nearby codons cognate with the deacylated P-site tRNA (Skabkin et al., 2013; Young et al., 2015), a mechanism enhanced by viral mRNA containing the *cis*-regulatory ‘termination upstream ribosomal binding site’ (TURBS) motif (Zinoviev et al., 2015). Thus, ribosome recycling by ABCE1 is a decisive branch point between 80S and 40S re-initiation. Interestingly, various subunits of the eIF3 complex have distinct effects on translation re-initiation suggesting a regulatory role for the ABCE1-eIF3 tandem. Thus, eIF3j/Hcr1, which assists ABCE1 during 80S recycling, reduces the efficiency of translation re-initiation *in vitro* (Skabkin et al., 2013). In contrast, eIF3h significantly promotes re-initiation in human cells (Hronova et al., 2017) and plants (Roy et al., 2010), while it strongly depends on eIF3a in yeast (Munzarova et al., 2011). Consistently, ribosome recycling is regarded as a regulatory gateway in canonical and aberrant translation (Dever and Green, 2012; Buskirk and Green, 2017; Gerovac and Tampé, 2019) and is strongly connected to ribosome homeostasis (Young et al., 2015; Mills et al., 2016).

However, the pathologic phenotypes of certain mutations involving ribosomal proteins or ribosome biogenesis factors cannot be explained by neither *cis*- nor *trans*-regulatory elements of translation (Tahmasebi et al., 2018). The most prominent example is Diamond-Blackfan

anemia (DBA), which affects different tissues depending on which ribosomal protein is defective (Draptchinskaia et al., 1999; Gazda et al., 2008; Boria et al., 2010; Gazda et al., 2012). These findings shift the ribosome into focus. The cellular pool of ribosomes is heterogenous and distinct populations vary in rRNA and ribosomal protein composition as well as their respective chemical modifications (Slavov et al., 2015; Ferretti et al., 2017; Shi et al., 2017). However, it remains controversial whether ribosome heterogeneity is actively regulated within the cell and reflects functional specialization leading to populations of ‘specialized ribosomes’ (Gilbert, 2011; Xue and Barna, 2012; Emmott et al., 2019; Ferretti and Karbstein, 2019).

Between 200 and 400 copies of rDNA exist in the human genome. Some of them carry mutations, which lie within the sequence of mature rRNA and exhibit tissue-specific expression (Parks et al., 2018). Additionally, over 200 post-transcriptional modifications concentrate in most important functional rRNA regions and were shown to participate in mRNA and tRNA binding as well as subunit association in Bacteria and Eukarya (Polikanov et al., 2015; Sharma and Lafontaine, 2015; Natchiar et al., 2017; Roundtree et al., 2017). They can alter the local structure of ribosomes, influence mRNA selectivity (Schosserer et al., 2015; Sloan et al., 2017; Sharma et al., 2018), and are thereby involved in the development of cancer (Bellodi et al., 2010; Truitt and Ruggero, 2017) as well as several hereditary diseases (Doll and Grzeschik, 2001; Armistead et al., 2009). Ribosomal subsets differing in protein composition can arise from mutations (Draptchinskaia et al., 1999), haploinsufficiency (Ebert et al., 2008), or tissue-specific expression of paralogous genes (Williams and Sussex, 1995; Marygold et al., 2007), prevalently in diseases, during development (Whittle and Krochko, 2009) or stress response (Zhang and Lu, 2009). Numerous ribosomal proteins affect specific translation of mRNA subpools (Kondrashov et al., 2011; Ferretti et al., 2017; Shi et al., 2017; Yamada et al., 2019) as reviewed elsewhere in more detail (Shi and Barna, 2015; Emmott et al., 2019). Additionally, over 2500 post-translational modifications of ribosomal proteins are known (Emmott et al., 2019), some of which contribute to translational selectivity, e.g. during mitosis (Imami et al., 2018) or in Parkinson’s disease (Martin et al., 2014). Considering the sophisticated biogenesis and degradation pathways, ribosome heterogeneity may also arise from stable ribosome-turnover intermediates (Ferretti and Karbstein, 2019). If so, it must be elucidated whether these intermediates are functional and to what extent their retention is regulated within the cell.

However, not only (i) mRNA selectivity of structurally diverse ribosomes may be crucial to induce a physiological effect but also their (ii) localization, (iii) accuracy, (iv) processivity, and (v) speed (Dinman, 2016). Additionally, the biosynthesis of functional proteins depends on co-translational recruitment of auxiliary factors (e.g. chaperones and protein modification enzymes), which is likewise affected by ribosome composition (Simsek et al., 2017). The most prominent example is RACK1/Asc1, which conducts various roles in translation and bridges to numerous cell signaling pathways (Gallo and Manfrini, 2015). Importantly, RACK1/Asc1 is crucial to trigger mRNA surveillance and RQC pathways on stalled ribosomes (Ikeuchi and Inada, 2016; Sitron et al., 2017; Sundaramoorthy et al., 2017; Juskiewicz et al., 2018; Ikeuchi et al., 2019). During starvation, RACK1/Asc1 is depleted from ribosomes (Baum et al., 2004). Thus, the cell responds to environmental conditions and may control pathways downstream of translation *via* ribosome composition.

In contrast to the specialized ribosomes, the ribosome abundance hypothesis describes the selective translation of certain subsets of mRNA as an effect of ribosome amount and concentration (Lodish, 1974; Mills and Green, 2017) that can be dynamically regulated by the ribosome recycling factors ePelota and ABCE1 during cellular differentiation (Mills et al., 2016; Khajuria et al., 2018). Further, ribosomes occupy approximately 20% of the cytosolic volume and were shown to regulate cellular biochemistry by low-affinity interactions with major metabolic enzymes but also molecular crowding and phase separation in the cytosol (Delarue et al., 2018). The ribosome abundance hypothesis is equally justified, especially in regard to translation regulation by supramolecular ribosome assemblies in polysomes and quality control pathways triggered by collided ribosomes. In respect of the latter, the abundance and activity of ribosome recycling factors is putatively crucial for the fate of the nascent chain and mRNA (see previous section). Effective mRNA decay by the NGD pathway as well as ribosome ubiquitination and RQC depend on the presence of at least two collided ribosomes (Simms et al., 2017; Ikeuchi et al., 2019). However, stalled ribosomes are efficiently split in the presence of ePelota (Dom34) *in vivo* (Sitron et al., 2017). Thus, we assume that reduced ribosome recycling activity (either global or local) would lead to ribosome collisions at transient pause sites and enhance NGD and RQC. In contrast, elevated levels of ribosome recycling activity would protect mRNA from effective decay by resolving stalled ribosomes before NGD is triggered. During oxidative stress, the supply of ABCE1 with the essential FeS clusters is inhibited, which globally affects translation (Alhebshi et al., 2012).

Consistently, ePelota as well as multiple factors of the NGD and NSD pathways are essential for oxidative stress tolerance, possibly reflecting the increased requirement to resolve stalled ribosomes (Jamar et al., 2017). Another convincing example of translation regulation by ribosome recycling is found during erythrocyte maturation. ABCE1 depletion causes 80S build-up in the 3' untranslated region (3'UTR) of the transcriptome (which is rescued by elevated levels of ePelota at initial differentiation stage) (Mills et al., 2016). Consistently, the di-ribosome recognition factor ZNF598 is absent from rabbit reticulocyte lysate, which allowed the formation of stable di-ribosomes for structural assessment (Juskiewicz et al., 2018). Presuming that human and rabbit proteome changes in a similar fashion during erythropoiesis, NGD and/or RQC induced by ribosome collisions upon specific downregulation of ribosome recycling would interfere with cellular function and were thus abolished by loss of ZNF598.

Finally, the two major approaches explaining the regulatory role of the ribosome are not mutually exclusive, and a sophisticated interplay of both with the translational *cis* and *trans* regulators most likely shapes the cellular protein composition with all its consequences (Tahmasebi et al., 2018; Dalla Venezia et al., 2019).

Future perspectives

Beyond ribosomal complexes with a defined architecture, a concept for translation regulation in membrane-less organelles emerges as a mechanism for spatial and temporal control of protein biosynthesis (Protter and Parker, 2016; Aguilera-Gomez and Rabouille, 2017; Franzmann and Alberti, 2019). Biochemical and physicochemical processes underlying cytosolic heterogeneity and their biological consequences are not yet understood and remain one of the most exciting future perspectives. Thus, translation regulation, mRNA homeostasis and co-translational modification, translocation, and folding of proteins must be studied in the light of the diverse cytosolic formations such as P-bodies, stress granules, or Sec bodies (Zacharogianni et al., 2014; Acosta-Alvear et al., 2018; Van Treeck and Parker, 2018). As the composition, stoichiometry, and organization of membrane-less organelles are not well defined, structural studies of cellular ensembles by cryo-electron tomography in combination with high-resolution cryo-EM will obviously be most valuable. Additionally, membrane-less organelles are highly dynamic, which is why biophysical methods with spatial and temporal resolution are inevitable in this novel field of research. Strikingly, RNA-protein granules are linked to development, neurodegenerative disorder,

and viral infection (Protter and Parker, 2016; Wolozin and Ivanov, 2019), emphasizing their physiological significance. In our current models, only mRNA and individual factors bind to isolated ribosomes and form defined complexes. In contrast to this, we tend to speculate that ribosomes create a molecular sociology around them and that factors therein compete with each other, thus establishing dynamic regulatory mechanisms including (anti-) synergistic effects. The molecular surrounding depends on structure, composition, and availability of the ribosomes, their localization, overall cellular condition, and many more yet elusive parameters. This model is in line with the hypotheses of the specialized ribosome (Xue and Barna, 2012) and ribosome abundance (Mills and Green, 2017) as well as the concept of molecular phase separation (Turoverov et al., 2019), and awaits validation or refutation by accurate *in vivo* experiments and visualization on single-molecule basis.

Acknowledgments: We thank Inga Nold and Andrea Pott for editing and Simon Trowitzsch, Lukas Susac, Holger Heinemann, Stefan Brüchert, and Bianca Hetzert for helpful discussions. E.N.G. was supported by the Christiane Nüsslein-Volhard Foundation, L'Oréal, and the United Nations Educational, Scientific and Cultural Organization (UNESCO) (funder Id: <http://dx.doi.org/10.13039/100005243>), Grant Number: Nürenberg-Goloub (Nüsslein-Volhard/L'Oréal Grant). The German Research Foundation (DFG) SFB.902 'Molecular mechanisms of RNA-based regulation' and the Cluster of Excellence EXC115 funded this work (to R.T.) (funder Id: <http://dx.doi.org/10.13039/501100001659>).

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