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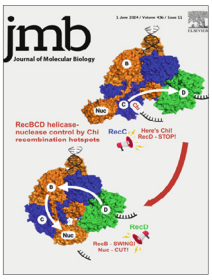

Membrane-associated mRNAs: A Post-transcriptional Pathway for Fine-turning Gene Expression

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Abstract

Gene expression is a fundamental and highly regulated process involving a series of tightly coordinated steps, including transcription, post-transcriptional processing, translation, and post-translational modifications. A growing number of studies have revealed an additional layer of complexity in gene expression through the phenomenon of mRNA subcellular localization. mRNAs can be organized into membraneless subcellular structures within both the cytoplasm and the nucleus, but they can also targeted to membranes. In this review, we will summarize in particular our knowledge on localization of mRNAs to organelles, focusing on important regulators and available techniques for studying organellar localization, and significance of this localization in the broader context of gene expression regulation.

Introduction

Gene expression is a fundamental and highly regulated process that utilizes genetic information to synthesize functional gene products, such as proteins and RNA, across all organisms. It involves a series of tightly coordinated steps, including transcription, post-transcriptional processing, translation, and post-translational modifications. These intricate processes work together to ensure the accurate and timely production of gene products, which are necessary for maintaining cellular physiological functions and the ability to respond to external stimuli.

In the past decades, a growing number of studies have revealed an additional layer of complexity in gene expression through the phenomenon of mRNA subcellular localization.[1] mRNAs are not evenly distributed in the cytoplasm. Indeed, they exhibit unique subcellular localizations to meet specific cellular physiological requirements.[1,2] The localization of mRNAs to the endoplasmic reticulum (ER) has been well characterized and extensively studied.[3–5] More recently, studies have also demonstrated that mRNAs can localize to mitochondria.[6] Limited research suggests that mRNAs also localize to peroxisomes and to the nuclear membrane[7,8] (Figure 1). Furthermore, mRNAs can be organized into membraneless subcellular structures within both the cytoplasm and the nucleus. Well-established examples of these structures include nuclear speckles, P-bodies, and stress bodies,[9,10] but additional less well characterized membraneless structures have been described.[11–13] What is particularly intriguing is that some of these structures have associations with cellular membranes.[11,14]

The subcellular localization of mRNAs plays a critical role in various biological processes. For instance, when mRNAs localize to the ER or

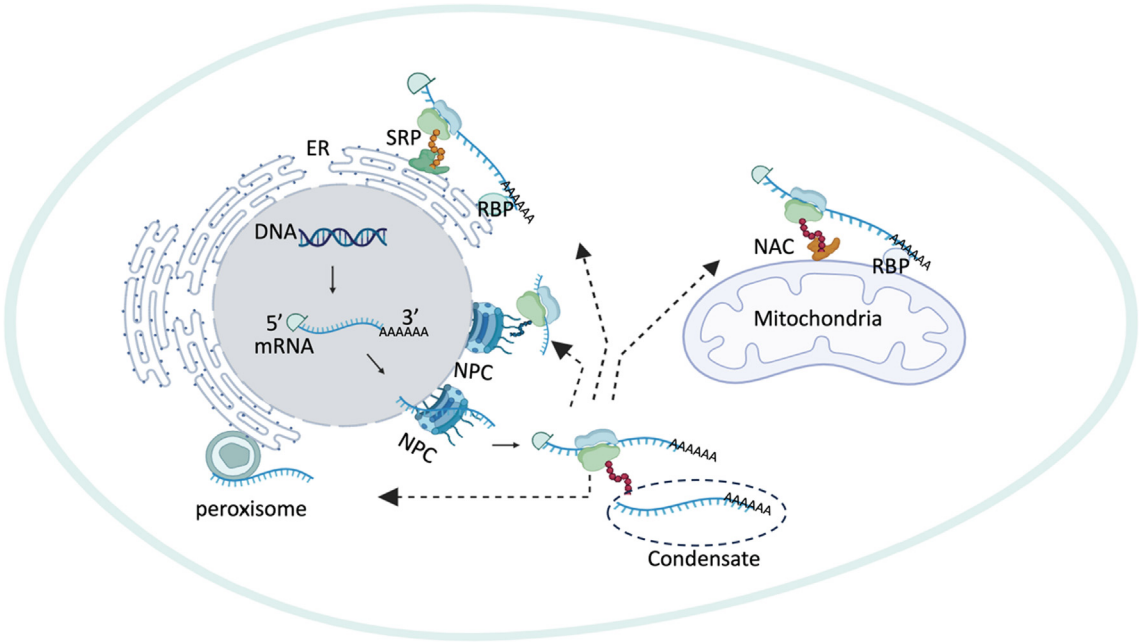

Figure 1. Cartoon illustration of the subcellular localization of mRNAs within a cell. DNA is transcribed into mRNA within the cell nucleus. During transcription mRNA is capped and receives a 3′ poly-A tail, then exits the nucleus through nuclear pore complexes (NPCs). In the cytoplasm, mRNAs can be translated or not. mRNAs that are not translated can be degraded or accumulate in condensates. Newly synthesized peptides resulting from translation bind to chaperones. RNA-binding proteins (RBPs) can interact with the 3′ UTR of mRNAs. mRNAs can travel along microtubules or microfilaments to reach various subcellular organelles. SRP: signal recognition particle; NAC: nascent polypeptide associated complex. The 60S ribosome particle is depicted in green and the 40S in blue. The relative sizes of components depicted in this cartoon are not drawn to scale.

mitochondria, this allows for co-translational import and minimizes the risks of misfolding of nascent proteins.[15] Moreover, the storage of mRNAs in specific subcellular compartments enables a rapid response to external stimuli, contrasting with the time-consuming process of *de novo* mRNA synthesis.[13] In fly embryos, mRNA localization is crucial for proper development. It contributes to the establishment of cellular polarity during asymmetric cell division.[16,17]

Some of the key regulatory factors involved in mRNA localization have been characterized, including N-terminal signal sequences, *cis*-acting elements within mRNA 3′ untranslated regions (UTRs), as well as *trans*-acting factors such as the signal recognition particle (SRP), mRNA binding proteins, ribosomes and the nascent polypeptide-associated complex (NAC)[18–20] (Figure 1). It is currently believed that mRNAs are transported along actin filaments or microtubules by the action of ATPase motors, although the precise mechanisms involved remain incompletely understood[21,22] and for reviews see.[23–25]

Understanding the mechanisms and physiological functions of mRNA subcellular localization expands our knowledge of gene expression regulation and the interplay between mRNA, protein synthesis and cellular organization. In this review, we will delve deeper into the intricate mechanisms of mRNA subcellular localization, in particular localization of mRNAs to organelles, its regulators, the available techniques for studying it, and its significance in the broader context of gene expression regulation.

Localization of mRNAs to Organelles

Organellar mRNA localization may occur independently of translation or during translation. It involves the recognition of mRNA or nascent peptide by molecular chaperones and co-chaperones. Subsequently, the mRNA or the ribosome-associated nascent peptide complex (RNC) may move along the cytoskeleton and bind to receptors on the organelle. The presence of the mRNA at the organelle in turn will facilitate the translocation of newly synthesized peptides from this mRNA to the organelle.

As will be outlined below, there are numerous observations suggesting co-translational targeting of mRNAs to organelles, but these findings are often suggestive rather than conclusive evidence of co-translational targeting and the field continues to grapple with many unanswered questions and challenges. Further research is required to

establish the complete chain of evidence, which should encompass: (i) Demonstrating the existence of organelle recognition elements on mRNA and/or RNC; (ii) Uncovering how these define localization of the mRNA to the target organelle, whether through autonomous mechanisms or interactions with receptors; (iii) Confirming the significance of these in various aspects of protein biosynthesis, such as translation, folding, assembly, import or stability; (iv) Validating the generality of this pathway, i.e., confirming that a substantial number of mRNAs utilize this mechanism. Nevertheless, because the evidence is accumulating it is worth summarizing the state of the field.

ER-associated mRNAs

mRNA targeting to the ER was the first to be identified and it has been extensively characterized in eukaryotes. While a majority of eukaryotic studies have been conducted in yeast, investigations in plants, flies and human cells, have expanded findings and validated that the mechanisms are conserved.

Classic SRP-mediated mRNA targeting to the ER. Early studies indicated that mRNAs were targeted to the ER co-translationally and relied on the signal recognition particle (SRP). SRP is a complex composed of both protein subunits and RNA.[26,27] This ribonucleoprotein complex can be subdivided into two distinct domains: the Alu domain, which plays a role in elongation arrest, and the S domain, responsible for the binding of the signal peptide and interaction with the SRP receptor (SR).

- *Signal sequence recognition by SRP*

The specific peptide sequence located at the N-terminus of polypeptides recognized by SRP is referred to as the signal sequence. It is believed that mRNA targeting begins during protein synthesis when the signal sequence emerges from the ribosome tunnel and can interact with the SRP. Although early understanding suggested that a core of 8–12 hydrophobic amino acids, forming an α-helix, was the major determinant for signal sequence recognition by SRP,[28] later studies indicated that hydrophobicity alone is not a sufficient determinant for SRP-dependence.[29] Indeed, the difference in hydrophobicity between SRP-dependent and SRP-independent signal sequences is relatively small, indicating the involvement of additional molecular features.[29] Ongoing research is still trying to define the features that contribute to specify SRP-dependence. The interaction between SRP and the nascent chain has a unique characteristic in eukaryotes. Unlike bacterial SRP, eukaryotic SRP, specifically the subunits Srp9/14 in budding yeast, can transiently arrest or slow down polypeptide chain elongation upon binding to the signal sequence as it emerges from the ribosome.[30,31] Cryo-electron microscopy analysis has shown that mammalian SRP forms an elongated kinked structure with its Alu domain, comprising the SRP9/14 heterodimer and extending into the elongation factor binding site of the ribosome.[32] It is thought that the Alu domain of SRP might delay the elongation cycle by preventing the binding of elongation factor eEF2. This interaction between SRP and the ribosome occurs during the translocation of tRNA from the A to the P-site in yeast and mammals, influencing the elongation process.[33,34]

- *Interaction between SRP and SR*

The interaction between the SRP complex and SR on the ER membrane is a crucial step in the process of nascent polypeptide translocation. This interaction is contingent upon the prior engagement of SRP with the signal sequence. The SR is composed of two subunits: SRα, a peripheral ER membrane protein, and SRβ, an integral membrane protein with a single transmembrane domain.[35,36] The interaction between SRP and SR involves complex formation, rearrangement, and dissociation, driven by GTP hydrolysis, ensuring the delivery of signal peptides and directed translocation of nascent peptides to the Sec61 translocon.[37–43]

- *The Sec61 complex mediates protein translocation*

The Sec61 complex serves as a protein-conducting channel responsible for either translocating proteins across the ER membrane or integrating them into the ER membrane.[44,45] When the RNC binds to the translocon, it triggers the opening of the channel. This event leads to a partial opening of the lateral gate and the dissociation of the RNC from SRP.[46–48] Besides its role as a pore in the ER membrane, the Sec61 complex also assists in the folding of transmembrane helices of new proteins in the hydrophobic lipid bilayer environment[49–52] (Figure 2**A).** A recent study showed that the multipass translocon contains a semi-enclosed lipid-filled cavity where multiple transmembrane domains of proteins are accommodated during their formation, challenging the understanding that these domains successively pass through a lateral gate of Sec61.[53]

SRP-independent mechanisms. The classic SRP mechanism to define targeting of mRNA to the ER has been regarded as well-established. However studies have increasingly demonstrated the existence of additional mechanisms that allow mRNAs to localize to the ER independently of translation, signal peptides and SRP.[54–56] Concerning the role of SRP, it was found that translation and nascent-chain dependent localization to the ER can also be mediated through interaction of the nascent

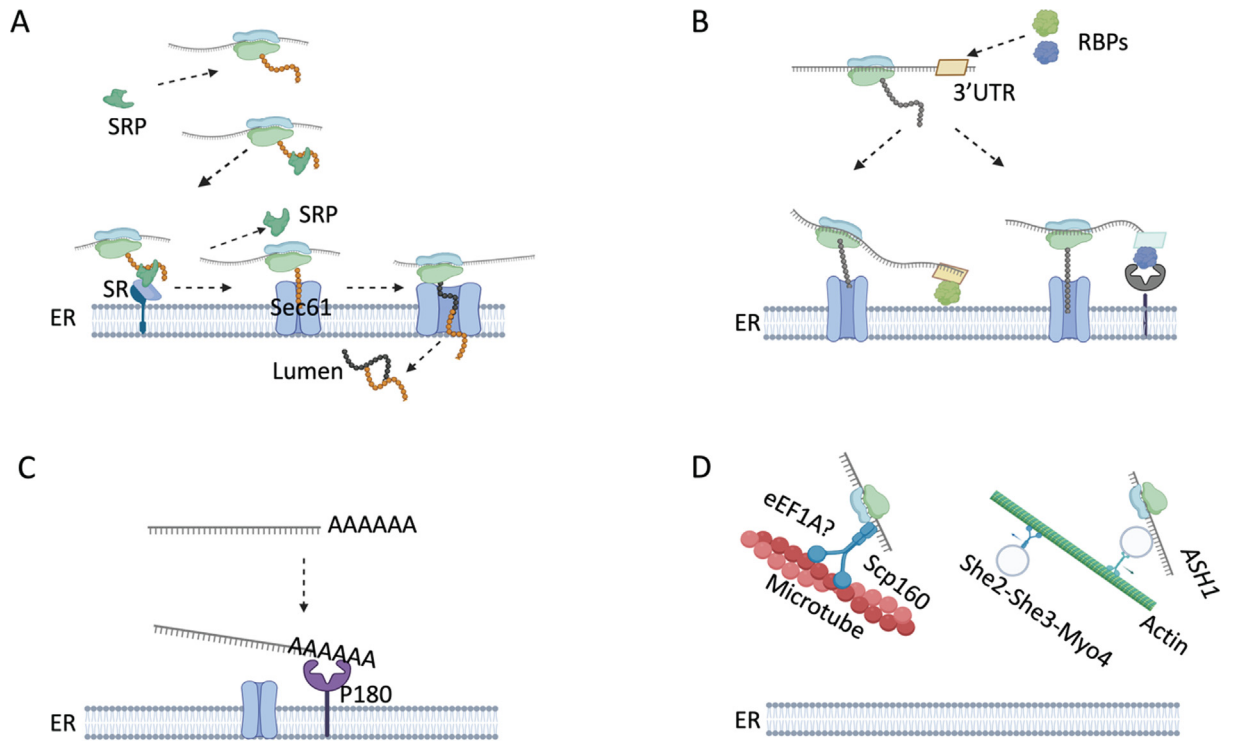

Figure 2. Carton illustration of mRNA targeting to the ER by different mechanisms. A: SRP translation-dependent mechanism. The binding of SRP to the nascent chain transiently inhibits translation. The SRP receptor (SR) on the ER membrane recognizes and transports SRP to the Sec61 translocon, releasing SRP from the nascent chain to allow translation to continue. The Sec61 complex mediates transport of proteins into the lumen and aids in their folding. **B: The SRP-independent pathway**. Distinct RNA-binding proteins (RBPs) recognize unique motifs within the 3′ untranslated region (3′UTR) of mRNAs, facilitating mRNA localization to the ER, either through recognition by ER receptors or by direct binding to the ER. **C: The ER membrane protein p180 pathway.** p180 directly recruits mRNAs to the ER through its interaction with the polyA tail of mRNAs. **D: Moving to the ER**. Various mRNAs, with the assistance of distinct RBPs, utilize motor proteins for energy to navigate along microtubules or microfilaments to reach the ER.

chain with a relevant partner at the ER, independent of a signal sequence. This is for instance the case for the Dia mRNA whose encoded N-terminal GTPase-binding domain can interact with active and *peri*-nuclear-ER RhoA.[54] Moreover, stable knockdown of SRP54 in HeLa cell lines resulted in defects in membrane protein processing but did not globally alter mRNA distribution patterns.[55] Furthermore, while immediate changes upon SRP depletion assays have indicated that SRP plays a general role in targeting proteins with transmembrane domains, on the contrary, many proteins containing cleavable amino-terminal signal peptides were efficiently targeted cotranslationally even in the absence of SRP.[57] Concerning the role of translation, Nicchitta and colleagues found that mRNAs retain physical interactions with the ER even after the disassembly of membrane-bound ribosomes using EDTA treatment. They also observed ER localization of the GRP94-encoding mRNA even in the absence of translation caused by mutations in the start codon, insertion of a 5′UTR stem-loop structure, or deletion of the encoded signal sequence. Finally, concerning the signal sequence, a study in yeast combining biochemical purification of ribosome populations and ribosome profiling showed that SRP preferentially binds secretory RNCs before their targeting signals are translated. Indeed, ribosome footprints from most SRP-bound monosomes were abundant well before translation of the first targeting signal. It was proposed that non-coding mRNA elements promote this signal-independent pre-recruitment of SRP.[56] Proximity-specific ribosome profiling also indicated that some proteins were able to engage the ER even before signal sequence emergence.[58]

3′UTR in cis and RBPs in trans. RNA binding proteins (RBPs) play a crucial role as post-transcriptional regulators of mRNA localization, translation, and degradation processes. A comprehensive analysis of yeast RBPs has identified specific RBPs, including Bfr1, Whi3, Puf1, Puf2, Scp160 and Khd1, which are associated with mRNAs encoding proteins that locate in various cellular compartments such as

the yeast cell wall, the plasma membrane and the ER.[59] The ER is the primary site responsible for the translation of membrane and secreted proteins. Subsequently, individual studies have confirmed the co-localization of Khd1 with the ER, and the dependency on Khd1 for the localization of *MID2* and *SLG1*/*WSC1* mRNAs to the ER, which occurs independently of translation.[60] Furthermore, in yeast it has been confirmed that Whi3 that binds *CLN3* mRNA, localizes to the ER and is needed for the efficient retention of cyclin Cln3 at the ER in early G1 cells. The mRNAs associated with Whi3 are enriched for clusters of the tetranucleotide GCAU and mutation of the GCAU clusters in the *CLN3* gene results in decreased association of its encoded mRNA with Whi3.[61]

The Pumilio-Fem-3-binding factor (PUF) proteins are a group of RBPs that are evolutionarily conserved.[62] Puf1 and Puf2 are members of the PUF protein family in yeast that are associated with mRNAs encoding membrane-associated proteins. Although it is not fully understood whether Puf1 and Puf2 directly target mRNAs to the ER, their association with mRNAs encoding membrane-related proteins suggests their potential involvement in SRP-independent mRNA targeting to the ER.[63,64] The human orthologs of yeast Puf1 and Puf2 recognize the UGUA(AUC)AUA motif in the 3′UTR of mRNAs. Both proteins exhibit a significant overlap in their target mRNAs that largely encode membrane proteins. Therefore, they are also considered to be involved in the transport of mRNAs to the ER.[63]

Scp160 is an RNA-binding protein with 14 tandemly repeated heterogenous nuclear ribonucleoprotein K-homology domains, which are involved in RNA binding.[65] Despite being present on cytosolic polysomes, Scp160 is primarily localized to the ER. Its association with the ER is dependent on the presence of mRNA and requires the function of microtubules but is independent of the actin cytoskeleton.[66]

She2 was also identified as an RBP that is necessary to coordinate mRNA localization and distribution to the ER during the budding process in yeast. It binds to *ASH1* mRNA and facilitates its movement along ER tubules to the growing bud. This interaction between She2, *ASH1* mRNA and ER tubules ensures the precise localization of the mRNA during cellular growth and division.[67]

In human cells, the STAU1 and STAU2 RBPs have been identified as necessary for mRNA transport along microtubule tracts in neuronal dendrites.[22] STAU1 co-localizes with the rough ER and was detected in membrane-bound polysome fractions.[68] In contrast, STAU2 only co-localizes with smooth ER in neurons and a fraction of STAU2 associates with free ribosomes.[69] A global screening approach determined that STAU1- and STAU2-targeted mRNAs are distinct but also overlap to a small extent. The majority of these target mRNAs are involved in cell metabolism, transport, transcription, regulation of cell processes, and catalytic activity.[70] Therefore, STAU1 and STAU2 are considered to be involved in mRNA transport to the ER.

It is worth noting that a single RBP can bind to multiple mRNAs, and conversely, the same mRNA can be targeted by multiple RBPs, indicating the presence of a complex network of interactions among RBPs (Figure 2**B**).

Not all of the mentioned RBPs reside at the ER, suggesting the presence of receptor proteins at the ER that facilitate the docking of the mRNAs to the ER. One potential candidate for such a receptor is an abundant ER protein p180. p180 is a mammalian membrane protein that contains a single transmembrane domain. It has been identified as a putative mRNA receptor involved in the direct binding of the mRNA poly(A) tail at the ER, primarily through its lysine-rich region (Figure 2**C**). Overexpression of p180 has been shown to enhance the association of mRNAs with the ER without specifically clarifying whether the recruited mRNAs were associated with specific RBPs.[71]

Role of the cytoskeleton and motor proteins in mRNA targeting to the ER. To enable mRNA targeting to the ER, in addition to RBPs, motor proteins and the cytoskeleton are also needed. In the case of *ASH1* mRNA in yeast, the protein She2, assisted by the motor protein myosin Myo4, plays a crucial role in facilitating its movement to the bud. Specifically, She2 directly binds to a zipcode within the *ASH1* mRNA and this helps to recruit the She3-Myo4 complex. She3 acts as a linker, connecting She2 to the motor protein Myo4. This interaction between She2, She3 and Myo4 enables the efficient transport of *ASH1* mRNA from the mother cell to the daughter cell along the actin filaments[21,72] (Figure 2**D**, right panel). Another study showed that the ER localization of Scp160 depends on the presence of microtubules. When microtubules were depolymerized using benomyl or nocodazole, Scp160 was completely redistributed from the ER to the cytosol within a short period. However, when the actin network was depolymerized using latrunculin A, the localization of Scp160 remained unchanged. These findings suggest that the accumulation of Scp160 at the ER is dependent on microtubules but independent of actin (Figure 2**D**, left panel).[66] The mechanism by which Scp160 anchors to microtubules remains unclear. One possible mechanism is through interactions with microtubule or actin-binding proteins. One such candidate could be the eukaryotic elongation factor 1A (eEF1A) that interacts with the translating ribosome and has been found to interact with both microtubules and actin.[73,74] This multifunctionality implies the potential for eEF1A to play a dynamic role in mRNA localization and transport processes that remains to be characterized. Cur-

rent studies have indicated that eEF1A binding to F-actin and aminoacylated-tRNA may be mutually exclusive.[75]

Ribosome pausing contributes to mRNA targeting to the ER. Several studies have revealed an additional mechanism for regulating mRNA targeting to the ER, namely ribosome pausing. Ribosome pausing refers to the prolonged residence of ribosomes on a specific codon during translation, and it is a conserved regulatory mechanism (for reviews see[76,77]). Diverse factors can contribute to ribosome pausing, including the mRNA sequence and structure, the availability of tRNAs, and the synthesis of the nascent peptide.[77,78] Prolonged ribosome pausing can lead to ribosome collisions of two to three ribosomes (ribosome dimers or trimers). Ribosome profiling analysis has shown that ER-targeted mRNAs exhibit ribosome-protected fragments that cover more abundantly the initial section of the mRNA, and an accumulation in particular of ribosomes over a GGC glycine codon located approximately 75 codons into the coding sequence. The location of this codon is such that RNCs paused at this codon will have the signal sequence exposed and available for SRP binding.[79] Disome sequencing, which examines collided ribosomes, has also demonstrated an accumulation of disomes over such located glycine codons.[80] This is consistent with the idea that SRP slows down translation or that slowing down the movement of ribosomes provides sufficient time for SRP binding to the signal sequence, and mRNA targeting to the ER.

A number of studies have indicated that the conserved Ccr4-Not complex plays a crucial role in the regulation of ribosome pausing.[81–84] In particular one study has suggested that in human cells CNOT1, the essential scaffold subunit of the Ccr4-Not complex, is required for the proper targeting of mRNAs to the ER. Specifically, it was shown that depletion of CNOT1 results in the reduction of ribosome occupancy downstream of the sequences mediating targeting of mRNAs to the ER as well as cytoplasmic accumulation of mRNAs normally located at the ER.[83] These findings suggest that ribosome pausing for mRNA ER localization is regulated by the Ccr4-Not complex. However, further research is needed to elucidate the precise regulatory mechanisms and factors involved.

Physiological and functional relevance of mRNA targeting to the ER.

- *Increasing the translation efficiency*

The translation of mRNAs into proteins occurs primarily on two populations of ribosomes: those free in the cytosol and those bound to the ER. Genome-scale studies have revealed that the mRNA transcriptome is broadly represented on the ER, with mRNAs encoding secretory and membrane proteins highly enriched on the ER.[85,86] However, mRNAs encoding cytosolic proteins can also localize to the ER, as supported by recent ribosome-proximity labeling,[87] and as exemplified by the *GAPDH* mRNA that encodes a cytosolic protein.[85] Interestingly, this mRNA can be found in both the cytoplasm and the ER subcellular fractions, where it associates with polysomes. The dual localization of *GAPDH* mRNA in both the cytoplasm and the ER significantly enhances its translation efficiency compared to when it is solely present in the cytoplasmic fraction.[88] This is consistent with studies using cell fractionation and polysome profiling, which have demonstrated that on average ribosomes are more densely loaded on ER-bound mRNAs compared to cytosolic mRNAs, indicating higher translation efficiency on the ER.[89] In addition, single-molecule quantification analyses have revealed that mRNAs encoding cytosolic proteins associate with the ER in a translation-dependent manner. These ER-associated transcripts exhibit higher ribosome occupancies compared to transcripts translated in the cytoplasm[90] (Figure 3**A**). These findings collectively suggest that mRNAs localized to the ER exhibit higher levels of expression compared to mRNAs localized in the cytoplasm. This underscores the role of mRNA localization as a regulatory mechanism influencing gene expression.

- *Co-translational folding*

Targeting of mRNAs to the ER represents an efficient way to minimize the risk of protein misfolding and aggregation. Indeed, it ensures that proteins are synthesized directly at the ER, where they can undergo co-translational translocation, reducing the chances of exposing hydrophobic domains of proteins leading to their aggregation in the hydrophilic cytosolic environment. Protein folding for ER proteins is generally considered to be co-translational, indicating that the mRNAs need to be transported to the ER.[51] Inefficient ER targeting can lead to pathological consequences as exemplified by the Prion protein (PrP). PrP possesses a signal sequence that supports only inefficient translocation into the ER, leading to the accumulation of a fraction of PrP in the cytoplasm and cytoplasmic toxicity. Providing PrP with a highly efficient targeting signal peptide can rescue mice from neurodegeneration caused by pathogenic PrP variants.[91] Therefore, maintaining efficient mRNA targeting to the ER is beneficial for the proper and effective folding of proteins, ensuring protein functionality. The process of co-translational folding of proteins at the ER typically involves three steps (Figure 3**B**): signal peptide cleavage, N-glycosylation, and folding mediated by resident chaperones in the ER.

Signal sequence cleavage: Signal sequences are usually found in the first ~25 amino acids of a protein and are composed of three domains: the

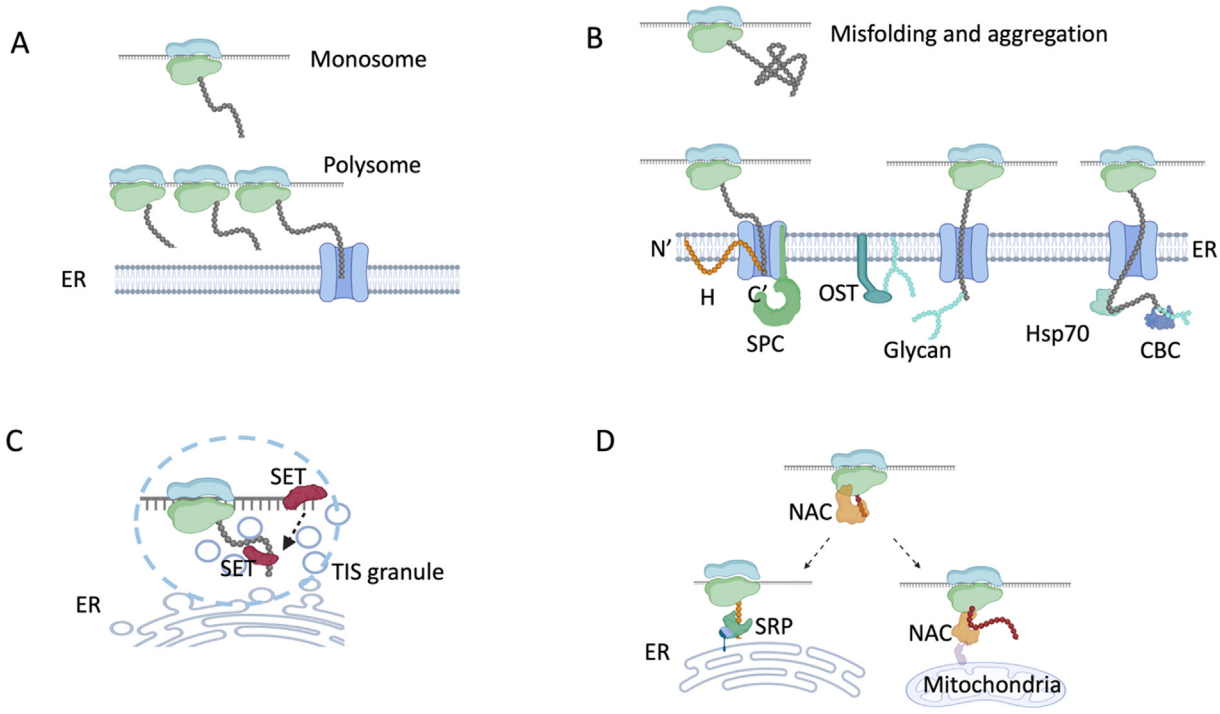

Figure 3. Cartoon illustration of alternative outcomes of mRNAs targeted to the ER. A: Translation efficiency. mRNA localization to the ER enhances translation efficiency by facilitating mRNA association with a higher number of ribosomes. **B: protein folding.** mRNA localization to the ER is necessary for proper protein folding and preventing protein aggregation. This folding process primarily includes signal peptide cleavage by SPC, N-glycosylation mediated by OST, and folding mediated by resident ER chaperones like Hsp70 or CBC. SPC: signal peptidase complex; OST: oligosaccharyltransferase; CBC: carbohydrate-binding chaperone. **C: TIS granules.** The RNA-binding protein TIS11B forms TIS granules that intertwine with the ER and facilitate the transfer of the effector protein SET from mRNAs to newly synthesized peptides. **D: NAC regulation.** NAC initially blocks SRP access to ribosomes during early translation, but recruits SRP as nascent chains emerge, preventing the mis-localization of ER-targeted mRNAs to mitochondria.

amino-terminal basic domain (N'-domain), the medial hydrophobic domain (H-domain), and the polar domain containing the cleavage site (C'-domain).[92] The N'- and H-domains of the signal sequence help to position the nascent polypeptide in a looped orientation during translocation, with the N-terminus facing the cytoplasm, the H-domain within the core of the lipid bilayer, and the C-domain facing the ER lumen.[93] The signal peptidase complex (SPC) recognizes the C-domain and cleaves the signal sequence from the nascent polypeptide.

N-Linked Glycosylation is a common post-translational modification of proteins in the eukaryotic secretory pathway. This modification involves the addition of carbohydrate moieties to specific Asparagine (Asn) residues within the consensus sequence Asn-X-Ser/Thr, where X can be any amino acid except proline. The addition of N-linked glycans typically occurs co-translationally, once the the Asn-X-Ser/Thr motif reaches approximately 13 amino acids deep into the ER lumen. The modification is catalyzed by the oligosaccharyltransferase (OST) complex, which transfers a preassembled carbohydrate composed of three glucoses, nine mannoses, and two N-acetyl glucosamines to the Asn residue.[94,95] The addition of bulky hydrophilic carbohydrate modifications alters the protein stability and conformation.

Chaperone-mediated folding: Protein folding in the ER is a complex process that requires the assistance of chaperones and enzymes housed within the ER. Two major chaperone systems are found in the ER: the classical chaperones and the carbohydrate-binding chaperone system. The classical chaperone system includes the chaperones of the Hsp70 family. These chaperones recognize immature, aberrant, or aggregation-prone proteins by identifying exposed hydrophobic segments that are normally buried in native proteins. They assist in the maturation of nonglycosylated proteins or unmodified domains on glycosylated proteins. The binding of chaperones is regulated by adenine-nucleotide binding and specialized cofactors. BiP in metazoans or Kar2 in yeast are Hsp70 family members and crucial chaperones in the ER. They bind to a wide range of proteins during their stay

in the ER and aid in their proper folding. BiP's binding cycle is controlled by nucleotide exchange factors (NEF) like BAP/Sil1 and GRP170, and J-domain proteins such as ERdj1, ERdj2, ERdj3, ERdj4, ERdj5, and ERdj6.[96,97] These chaperones play diverse roles in the ER, contributing to the folding and maturation of nascent chains and preventing aggregation and protein turnover. The carbohydrate-binding chaperone (CBC) system involves lectin chaperones like calnexin and calreticulin.[98] Calnexin is a membrane protein, while calreticulin is its soluble paralog[99]. They stabilize folding events in a domain-specific manner, prevent aggregation and turnover, retain nonnative substrates in the ER for additional folding attempts, facilitate disulfide bond formation mediated by the protein disulfide isomerase (PDI),[100] and potentially assist in proline isomerization, promoted by the prolyl peptidyl *cis–trans* isomerases (PPIases).[101]

It remains an open question whether all ER-associated proteins undergo co-translational folding. Indeed, ER also supports post-translational protein import and translocation of an unfolded protein, indicating that folding can occur with post-translation translocation.[102,103]

- *Promote localized protein–protein interactions*

Besides avoiding protein aggregation, mRNA localization to the ER can promote protein–protein interactions during translation. The existence of a specific subcellular compartment in close proximity to the ER known as the TIGER domain has recently come to light. It is a membraneless organelle formed by the RNA-binding protein TIS11B. The TIS11B granules intricately intertwine with the ER, selectively enriching or excluding specific mRNAs and proteins. This segregation enables the translation of particular mRNAs within a distinct ER subdomain. Moreover, the association of the TIGER domain with the ER plays a role in facilitating the transfer of the effector protein SET from mRNAs to newly synthesized peptides, a process dependent on their 3′UTR region, highlighting the crucial role of the ER in enabling specific protein–protein interactions and the dynamic regulation within the cell[14] (Figure 3**C**).

Mechanisms to avoid protein mis-localisation. Given the physical interaction between mitochondria and the ER,[104] ER proteins can be mis-localized to mitochondria, and vice-versa, and this can potentially lead to cellular dysfunction. Several mechanisms exist to protect from this. On one hand, SRP is assisted by another chaperone, NAC, a heterodimer that comprises an alpha subunit (Egd2 in yeast) and a beta subunit (Egd1 or Btt1 in yeast).[105] NAC serves as a universal co-translational chaperone that associates with nascent chains at the exit tunnel of cytosolic ribosomes.[106] During the early stages of translation, NAC is abundantly present and binds to most ribosomes via a high-affinity anchor, effectively blocking SRP access to emerging nascent polypeptides. Once nascent chains emerge, NAC recruits SRP, bringing it in close proximity to the ribosomal tunnel exit, thereby allowing SRP to recognize the ER signal sequences and efficiently guiding the nascent polypeptide to the ER membrane for proper translocation[107] (Figure 3**D**). Cryo-electron microscopy studies reveal that the core globular domain of NAC obstructs SRP from binding to ribosomes lacking an ER-targeting signal, while a flexible domain transiently captures SRP, allowing it to scan the emerging nascent chains. When an ER-targeting signal emerges, the globular domain of NAC becomes destabilized, enabling SRP to effectively bind to the nascent chain.[107] This mechanism contributes to ensuring the correct localization of proteins to ER or mitochondria during the translation process. Depletion of SRP results in ER mRNAs targeted to mitochondria, causing mitochondrial dysfunction.[57] However, ER proteins mislocalized to the mitochondrial membrane can be extracted by the Msp1 AAA-ATPase and this then facilitates their transfer to the ER.[108] Conversely, the ER-SURF pathway (ER surface-mediated protein targeting) rescues mistargeting of some mRNAs encoding mitochondrial inner membrane proteins to the ER, resulting in the presence of the encoded protein at the ER (exemplified by Oxa1). Via this pathway relying on the Djp1 co-chaperone, the protein can be rerouted and delivered to the mitochondria.[109]

Stress response. During protein folding in the ER, errors or misfolding can occur, leading to protein aggregation and the formation of aggregates. This can result in cytoplasmic toxicity, impact cell function and survival and trigger cellular stress responses. To counteract potential harmful effects, the ER has developed essential quality control mechanisms that ensure proper protein folding and prevent the accumulation of misfolded proteins. One of the critical quality control mechanisms in the ER is the unfolded protein response (UPR).[110] In yeast, the UPR is primarily regulated by the ER transmembrane receptor kinase/endonuclease Ire1, which initiates a signaling cascade upon sensing ER stress. It splices *HAC1* mRNA, leading to the translation of spliced *HAC1* mRNA. This occurs at specific foci on the ER membrane formed by stress-induced Ire1 oligomerization. The protein product Hac1 then acts as a transcription factor that induces the expression of stress response genes[111,112] (Figure 4**A**).

As mentioned earlier, ribosome pausing plays a role in mRNA targeting to ER. The ribosome associated quality control (RQC) has been well established as a co-translational quality control

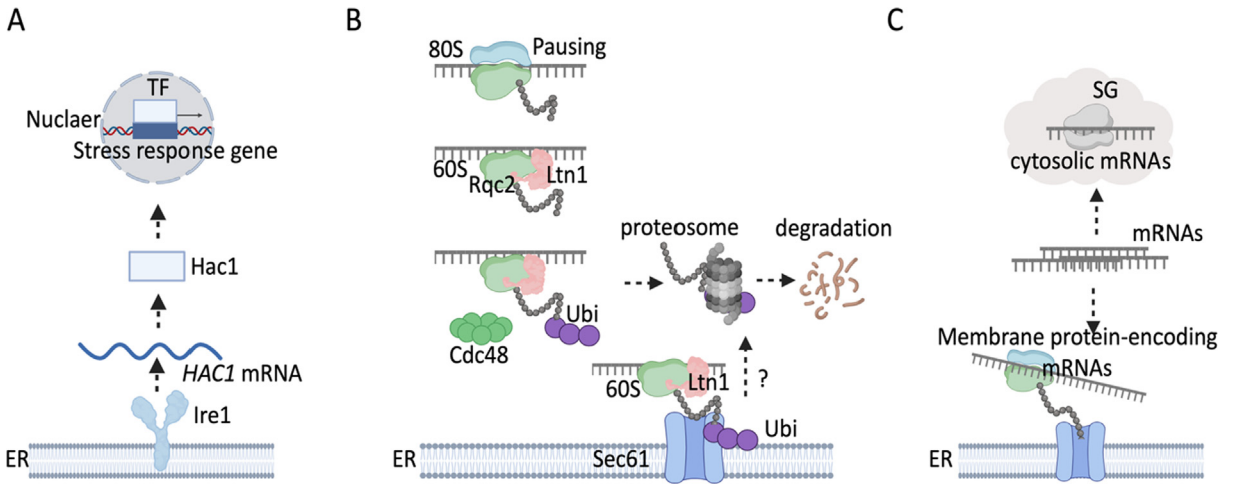

Figure 4. Cartoon of the ER-associated Stress response. A: The Unfolded Protein Response (UPR). UPR is initiated by the ER transmembrane kinase Ire1, which promotes splicing of the *HAC1* mRNA, enabling its translation and Hac1 protein nuclear relocation, where it functions as a transcription factor (TF) inducing the expression of stress response genes. **B: Ribosome-associated quality control (RQC).** RQC is triggered by ribosome pausing followed by ribosome collisions and can also occur for ER-localized mRNAs. Following 80S ribosome dissociation, Rqc2 recognizes 60S ribosome nascent chain complexes and recruits the E3 ubiquitin ligase Ltn1. Ltn1 ubiquitylates the nascent peptide and the peptides extracted with assistance by the Cdc48 ATPase are directed to the proteasome for degradation. **C: Avoiding the general stress response.** During stress, the majority of mRNAs encoding cytosolic proteins condensate in stress granules (SG) and are translationally silent, whereas translation of mRNAs encoding membrane proteins targeted to the ER is preserved.

mechanism, responsible for dealing with excessive ribosome pausing or stalling during translation. Briefly, when extensive ribosome pausing occurs, resulting in ribosome collisions, the 80S ribosome dissociates, leaving the peptidyl-tRNA retained by the 60S subunit. RQC components, including Rqc2, recognize these 60S ribosome nascent chain complexes (RNCs) and recruit the E3 ubiquitin ligase Ltn1 (Listerin in human). Ltn1 ubiquitylates the nascent peptide by targeting its N-terminal lysines.[113,114] After ubiquitination, the Cdc48 ATPase complex, along with Vms1 (a tRNA hydrolase), facilitates the extraction of the ubiquitinated proteins from the 60S.[115–117] These extracted proteins are then targeted to the proteasome for degradation. Additionally, the mRNA at the stalled ribosome is endonucleolytically cleaved near the site of pausing, generating 5′ and 3′ mRNA decay intermediates, which are further degraded by exoribonucleases. This mechanism is referred to as no go decay (NGD). It has been demonstrated that the RQC pathway occurs not only in the cytoplasm but also at the ER (reviewed in[118]) where an important fraction of all cellular translation occurs. Indeed, a little over one-fourth of all genes encode membrane proteins,[119] with the majority being translocated into the ER co-translationally.[3] Consequently, on average, about one-fourth of all translation stalls are likely to occur on ribosomes engaged at a translocation channel. In addition, it has been proposed that ER-associated ribosome quality control monitors in particular synthesis of polytopic membrane proteins, detecting misfolding events to prevent toxic protein accumulation[120] (Figure 4**B**). Moreover, a study performed in mammalian cells found two crucial components of RQC, Listerin and NEMF, associated with translocon-engaged 60S ribosomal subunits located on the ER. For RQC, the nascent polypeptides engaged with the translocon must undergo polyubiquitination and hence be exposed to the cytosol. This occurs at the ribosome-Sec61 junction. The E3 ligase Listerin accesses these nascent polypeptides through a gap in the ribosome-translocon junction, located in close proximity to the Sec61 lateral gate. Notably, the extraction and degradation of the polypeptide chain are less well understood mechanisms, possibly involving the ER-associated degradation (ERAD) machinery.[121,122]

Besides allowing to effectively repress protein synthesis by RQC as a stress response in case of increased ribosome stalling that might occur at the ER, increasing ER targeting of mRNAs can be a pathway to respond to stress. For instance, in response to cytotoxic stress in mammalian cells, various signaling cascades lead to the phosphorylation of translation initiation factor 2α, causing most cellular mRNAs to accumulate in stress granules (SGs) containing TIA-1, a nuclear RNA-binding protein.[123,124] This leads to significant impairment of protein synthesis. While the cytosolic mRNAs are sequestered into SGs, the ER-bound transcripts seem to escape this process (Figure 4**C**). These findings are crucial for cell survival, as many

membrane proteins translated at the ER play important roles in detoxification.[125]

Hence, mRNAs localized to ER exhibit the capacity for both upregulation and downregulation in response to various forms of stress.

Mitochondria associated mRNAs

In recent decades, there has been increasing attention on the investigation of mitochondrially-localized mRNAs and the regulatory factors involved in this process. The current understanding of mRNA localization to mitochondria is not as comprehensive as that to the ER. Classical techniques utilized for studying ER mRNA localization, but also newly developed methods, have been used to characterize mitochondrial mRNA localization. These combined efforts have significantly enhanced our knowledge of how mRNAs are targeted to mitochondria and allowed to characterize the factors responsible for mediating this localization.

Early studies using in situ hybridization combined with electron microscopy revealed the presence of two nuclear-encoded mRNAs associated with mitochondria in rat hepatocytes. Specifically, the mRNA encoding the F1 beta subunit of the ATPase was observed to be clustered near mitochondria.[126] Comprehensive investigations in yeast using differential centrifugation, filter arrays, and microarrays globally identified mRNAs associated with the mitochondrial fraction or that tended to localize near mitochondria. They revealed that mRNAs encoding inner membrane proteins, particularly those encoding components involved in the electron respiratory chain, were highly enriched in the mitochondrial fraction.[127–129] *In vivo* tagging methodologies in yeast, which involved fusing MS2 coat-protein binding sites to mRNA 3′-UTRs, provided additional evidence for the localization of mRNAs such as *ASH1*, *SRO7*, *PEX3*, and *OXA1* near mitochondria in living cells.[130] Moreover, RNA fluorescent in situ hybridization (FISH) was also employed to detect endogenous transcripts and further confirmed the association of mRNAs with the mitochondrial outer membrane.[131] The advancements in proximity-specific ribosome profiling have also enabled the isolation and characterization of mRNAs translated by mitochondria-associated ribosomes, with however only very few subsequent studies addressing the mechanisms uncovered in this work.[132]

Key players in mRNA localization to mitochondria. Several factors have been identified to play essential roles for localized translation near mitochondria (Figure 5**A**). One of the key elements is the mitochondrial targeting sequence (MTS), which has been characterized as a regulatory component facilitating mRNA localization to the mitochondria by Weiner and his colleagues.[6] In their study, they attached an MTS to the N-terminus of eGFP and an ER targeting signal to its C-terminus, resulting in a dual-signal eGFP that exclusively localized to mitochondria in HeLa cells. This finding highlighted the importance of co-translational import in directing proteins to the mitochondria.[6] Recent research has provided further insights into the mechanism of mRNA localization to mitochondria in a translation-dependent manner. The interaction between the MTS and the mitochondrial outer membrane receptor Tom20 has been found to be crucial for many mRNA associations with mitochondria. Structurally, the cytosolic domain of Tom20 adopts an α-helical conformation with a groove that accommodates the presequence peptide. Upon binding, the presequence peptide forms an amphiphilic helical structure where hydrophobic leucines are aligned on one side, facilitating their interaction with a hydrophobic patch in the Tom20 groove.[133–136] Although the deletion of Tom20 reduces mRNA association, it is important to note that mRNA localization is not completely abolished, indicating the involvement of additional factors associated with coding regions.[137]

NAC has emerged as mediating a pathway that involves the association of ribosome-nascent-chain (RNC) complexes with mitochondria in yeast. NAC associates with nascent chains at the exit tunnel of cytosolic ribosomes, facilitating their targeting to mitochondrial. It plays a vital role in segregating nascent chains from the cytosol until they reach a length of approximately 30 amino acids, thus preventing protein degradation and misfolding.[138,139] As mentioned above, during this process, NAC prevents SRP from accessing ribosomes that encode cytosolic and mitochondrial proteins, and this contributes to ensure the correct location of mitochondrial proteins.[140–142] NAC directly interacts with a positively charged amino acid cluster in the MTS of specific mitochondrial precursors, such as Oxa1 and Fum1.[143] NAC-associated nascent chains can dock to the mitochondrial outer membrane protein Om14, which interacts with another mitochondrial outer membrane protein Om45.[144,145] Deletion of Om14 results in reduced NAC association with mitochondria and decreased mitochondria-RNC association. The interaction between NAC and Om14 provides a physical mechanism for the co-translational import model. It is important to note that there is no Om14 homologue in higher eukaryotic organisms, raising questions about the conservation of this mechanism. In addition to its interaction with Om14, NAC can also dock on mitochondria via Sam37, but a possible association between Om14 and Sam37 remains unclear.[143,146] The interaction between NAC and the mitochondrial outer membrane potentially facilitates the transfer of the nascent chain to the major receptor of the import channel, Tom20. However, the precise mechanism by which NAC transfers the MTS to Tom20 remains

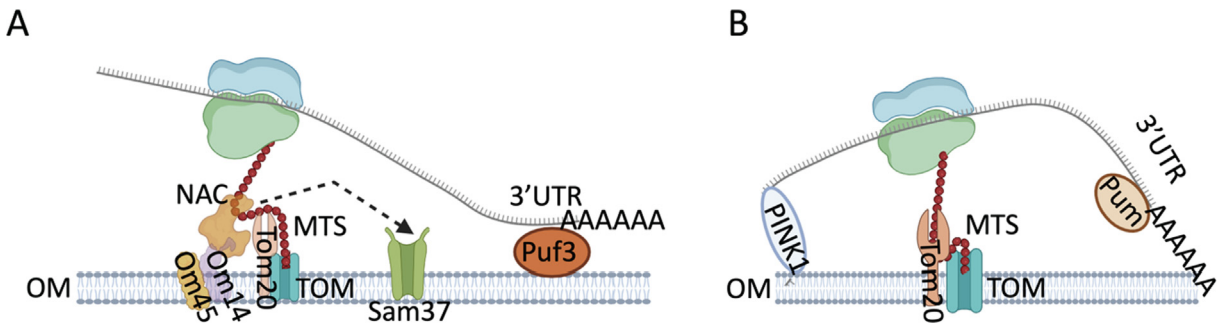

Figure 5. Cartoon illustration of the mechanism of mRNA localization to mitochondria in yeast and humans. A: Localization of mRNAs to mitochondria in budding yeast. In yeast, translation-dependent localization of mRNAs to mitochondria involves the mitochondrial targeting signal (MTS) that can be recognized by NAC and bound by the mitochondrial outer membrane receptor Tom20. NAC docks onto mitochondria by its interaction with the mitochondrial outer membrane proteins Om14, and its partner protein Om45, or Sam37. Additionally, mRNAs can be targeted to mitochondria through their 3′ untranslated region (3′UTR) by the RNA-binding protein of the pumillio family Puf3 in a translation-independent manner. **B: Localization of mRNAs to mitochondria in human**. In human, the mitochondrial outer-membrane kinase PINK1 directly recruits mRNAs to mitochondria in a translationally-repressed state. Repression is relieved through interactions with Parkin and subsequent removal of translation repressors, such as the pumillio family RNA-binding protein Pum. The mitochondrial receptor Tom20 plays a crucial role in mRNA localization, presumably through its interaction with the incoming MTS.

to be clarified. It is important to note that mitochondrial proteins are targeted to distinct subcompartments within the mitochondria through the use of various targeting sequences. For instance, proteins destined for the mitochondrial matrix and the inner membrane typically possess cleavable targeting sequences at their N-terminus. In contrast, outer membrane proteins are characterized by the presence of an uncleavable targeting signal located within the open reading frame. The different targeting sequences correspond to different mechanisms for directing proteins to their specific mitochondrial destinations.[147] Which targeting sequences mediate co-translational anchoring and whether some or all use the NAC-Om14 interaction for co-translational tethering of RNCs to the mitochondria is still an open question.

Extensive research has demonstrated the significant role of the 3′ UTR in mRNA localization to the mitochondrion, with a particular emphasis on the RBP Puf3 in yeast. Puf3 interacts with specific RNA motifs on mRNA 3′UTRs and can localize the target mRNAs to the mitochondria. Deletion of Puf3 reduces the mitochondrial localization of multiple mRNAs. Moreover removing the Puf3 binding site in the 3′UTR of the *BCS1* mRNA results in a decrease in its mitochondrial localization.[148] These findings highlight the crucial role of Puf3 in mediating mRNA localization to the mitochondria through its interaction with the 3′UTR. Although the transport of mRNAs to mitochondria may involve the cellular cytoskeleton and specific motor proteins, the precise mechanisms remain incompletely understood. In mammals, CLUH has emerged as another key RBP involved in regulating the transport and translation of nuclear-encoded mitochondrial mRNAs.[149] Its orthologue in flies, CluA/1, has been observed to form cytosolic granules that partially associate with tyrosinated tubulin and mitochondria.[150] Additionally, CluA/1 depletion disturbs the mitochondria network organization.[151] CLUH cross-linking immunoprecipitation experiments revealed mRNAs encoding mitochondrial respiratory chain proteins, molecules related to the tricarboxylic acid (TCA) cycle and proteins involved in fatty acid and amino acid metabolism.[149] The relevant binding motifs on these mRNAs have not been identified. Additional proteins have been discovered to function as RBPs and serve as anchors on the outer mitochondrial surface in different organisms, dependent or not, upon translation[152] and for review see.[153]

As discussed above, ribosome pausing plays a crucial role in directing the localization of mRNAs to the ER. A similar phenomenon has been observed in the context of mitochondria. A recent study conducted in yeast has revealed that ribosome pausing occurs downstream of the MTS in the *TIM50* mRNA. The pausing is facilitated by a sequence of seven consecutive proline codons. The consequence of this prolonged ribosome pausing is an increased likelihood of MTS recognition by the mitochondria, which, in turn, promotes the association of the mRNA with the mitochondrial surface. When these polyproline residues were deleted, it resulted in impaired mRNA localization, particularly under fermentative conditions. Additionally, slowing down ribosome movement in the *ATP3* mRNA by introducing a polyproline stretch led to an increase in the localization of the mRNA to the mitochondria.[154] Addtionally, a recent study in yeast has indicated

ribosome pausing on the *MMF1* mRNA downstream of the MTS-encoding sequences related to codon optimality, and necessary for optimal targeting of the mRNA to the mitochondrion.[84]

Studies in higher eukaryotes have identified the involvement of the mitochondrial outer membrane kinase PINK1 in mRNA localization to the mitochondrion (Figure 5**B**). Specifically, PINK1 binds to mRNAs encoding respiratory chain complex subunits and is implicated in their localization to mitochondria in collaboration with Tom20. These mRNAs are maintained in a translationally repressed state near mitochondria, and their repression is relieved upon association with the Parkin protein, leading to the subsequent release of repressors such as the PUF family protein, PUM.[155] The exact role of PUM, like that of Puf3 in yeast, in mRNA localization, is still not fully understood, in particular if and how they are anchored to the outer membrane.

It is important to highlight that the requirement of each pathway for mRNA localization at mitochondria, dependent on translation or mediated by the RNA and RBPs, varies depending on the specific mRNAs. Interestingly, the deletion of individual factors, such as Puf3 or Tom20, has only a minor impact on yeast growth under respiratory conditions. However, when both Puf3 and Tom20 are deleted simultaneously, it completely inhibits yeast growth. This observation underscores the presence of redundant pathways that collectively play a crucial role in targeting mRNAs to mitochondria.[137] The existence of redundant pathways suggests a level of flexibility and complexity in the regulation of mRNA localization to mitochondria. Different mRNAs may utilize alternative or overlapping mechanisms to achieve mitochondrial localization. This redundancy in the targeting pathways likely serves as a robust system to ensure the accurate localization and timely synthesis of mitochondrial proteins, which are essential for maintaining mitochondrial function and cellular energy metabolism.

Physiological and functional relevance of mRNA targeting to the mitochondria. Generally speaking the physiological and functional relevance of mRNA targeting to the mitochondrion is going to be similar to what was described above for the ER, with nevertheless some specificities.

- *Co translational import and complex assembly*

Localization of mRNAs at mitochondria plays a crucial role in various physiological processes, with one of its primary functions being the facilitation of protein import. By localizing mRNAs near the mitochondrial surface, the nascent polypeptide chains can be promptly directed to the translocation machinery, thus minimizing the risk of their aggregation or mistargeting in the cytoplasmic environment.[132,156–158] A model for co-translational import originated from the work on transfected HeLa cells using a dual signal approach mentioned above. In that study, researchers fused different MTS sequences from Otc or arginase II, to the N-terminus of eGFP and added ER or peroxisomal signals to the C-terminus (Figure 6**A**). The rationale was that if the free preprotein remained in the cytosol, it could be distributed to ER or peroxisomes through a post-translational pathway. However, the resulting fusion proteins were exclusively imported into the mitochondria, suggesting that co-translational import occurred. This outcome was further supported by the translocation of native preAldh, preOtc, and rhodanese, each with C-terminal ER or peroxisomal signals, which were also found to be translocated exclusively to the mitochondria, indicating the existence of a co-translational import pathway for these native proteins.[6] An alternate explanation for these results that should be kept in mind is that it could be that the signal was not functional at the C-terminus. However, the conclusion of co-translational import is additionally supported by *in vitro* experiments that have provided evidence that Fumarase undergoes co-translational import. In these specific *in vitro* experiments, the inhibition of mRNA translation lead to a significant reduction in the proportion of fumarase that entered the mitochondria.[159] In another *in vitro* study focusing on the import of yeast mitochondrial malate dehydrogenase(Mdh1) into mitochondria, EDTA was introduced into the *in vitro* assay, resulting in a substantial decrease in the proportion of matured Mdh1.[160] Clearly as outlined above, the evidence for co-translational import into mitochondria is less well-established when compared to co-translational import into the ER. Indeed, if NAC interacts with the mitochondrial outer membrane protein Om14,[144,161] how the nascent polypeptide chain is then delivered to the translocase or the receptor of the translocase remains to be determined.

Co-translation protein complex assembly can also be promoted by tethering of mRNAs to the mitochondria. It has been determined that mitochondria-associated mRNAs primarily encode inner membrane proteins, many of which are components of protein complexes.[128] Furthermore, mitochondrial DNA encodes essential subunits of the respiratory chain complexes for which some of the subunits are encoded by nuclear genes.[162] In yeast for instance, the ATP synthase comprises three mitochondrial DNA-encoded subunits and 14 nuclear-DNA encoded subunits, highlighting the need for a coordinated assembly process. Available data suggests that the assembly of the ATP synthase and cytochrome *c* oxidase likely occurs concomitantly with translation. Specifically, subunits encoded by mitochondrial DNA, such as Atp6p and Atp9p, are synthesized in close proximity to the inner mitochondrial membrane. Additionally,

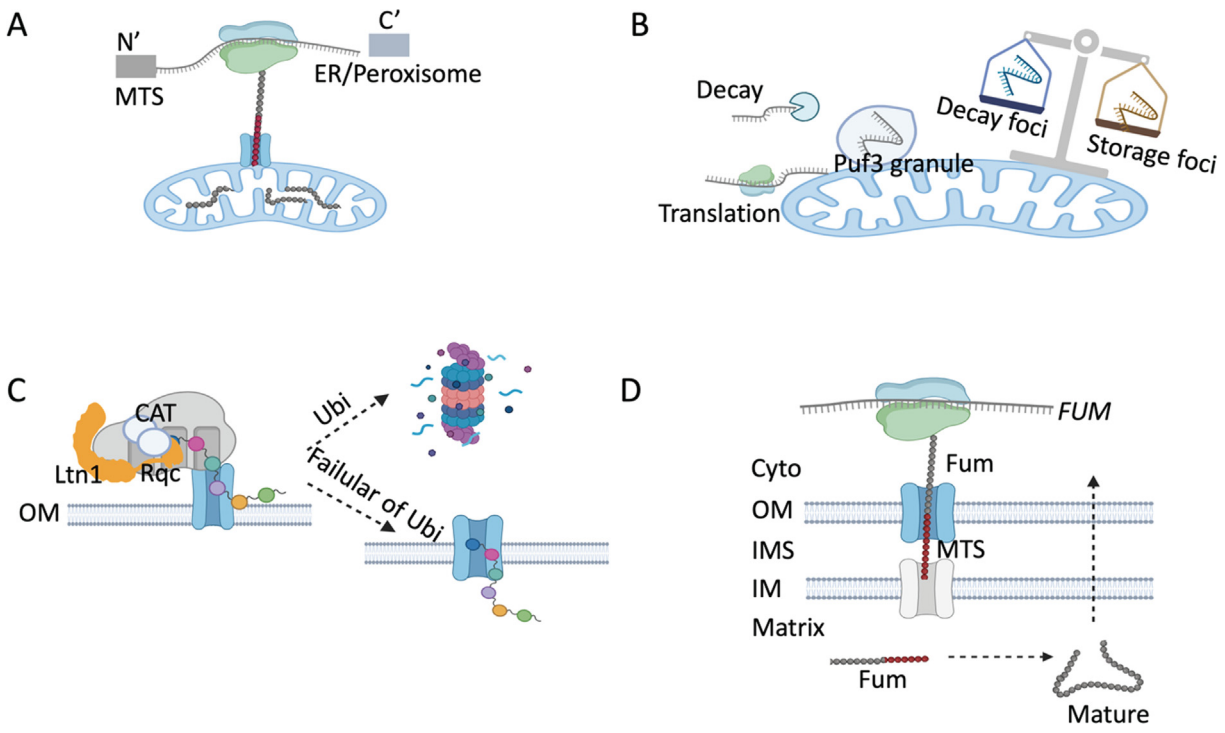

Figure 6. Cartoon illustration of the the outcomes of mRNA localization at mitochondria. A: Co-translational import. Co-translational import is demonstrated by the exclusive detection in mitochondria of a protein with a fusion of the mitochondrial targeting signal (MTS) at the N-terminus (N') and that of an ER or peroxisomal targeting signal at the C-terminus (C'). **B: Regulation of mRNA storage and stability**. In yeast during fermentation nuclear-encoded mRNAs can be stored in Puf3 granules or undergo degradation through Puf3-mediated pathways. Upon switch to respiration, Puf3 granules dissolve, releasing stored mRNA for rapid translation in response to the cellular demand. In *C.elegans*, mRNAs can accumulate in degradation or storage foci. Balanced regulation of these foci is crucial for responding to mitochondrial stress. **C: Co-translational quality control.** There is a surveillance mechanism during translation at mitochondria when ribosomes pause. It can be either cytoplasmic RQC, with ribosome dissociation, and Rqc2 recognizing 60S subunits, recruiting Ltn1, which then ubiquitinates nascent peptides, leading to their cytoplasmic degradation by the proteasome. Alternatively, if proteins are already engaged in import channels, Vms1 blocks Rqc2 and CAT-tailing (addition of a carboxy-terminal alanine and threonine tail through a noncanonical elongation reaction), promoting translocation of stalled nascent peptides into the mitochondria where they will be degraded. **D: Protein dual localization**. Fumarase (Fum) undergoes co-translational import into the mitochondrial matrix, where its MTS is cleaved, enabling it to fold into mature Fum. Partially matured Fum can return to the cytoplasm. OM: Outer membrane; IM: Inner membrane; IMS: Inter membrane space; Cyto: Cytoplasm.

nuclear-encoded proteins, including Aep2p/Atp13p, Atp10p, and Atp22p, are also translated in the vicinity of mitochondria. Regarding the assembly of cytochrome *c* oxidase, the process initiates with the mitochondrial translation of specific mRNAs, facilitated by nuclear-encoded translational activator proteins. These activator proteins are believed to be organized on the inner mitochondrial membrane's surface, ensuring the spatial co-localization of the synthesis of key subunits such as Cox1p, Cox2p, and Cox3p. This spatial proximity enhances the efficiency of the assembly process.[163] However, further experimental evidence is required to fully support these findings.

- *mRNA storage and stability*

In yeast, the demand for mitochondria varies with environmental changes. When glucose is available and used as the carbon source, yeast mainly undergo fermentation and require fewer mitochondria. However, under glucose starvation conditions, mitochondria are needed for respiration to sustain cell survival, leading to an increased demand for mitochondria. To rapidly respond to environmental changes, the cell nucleus continuously produces mRNAs encoding mitochondrial proteins, even under low demand conditions. Excess mRNAs are partially degraded, while some are stored. Interestingly, both of these processes can be mediated by Puf3. For instance, when glucose is the carbon source, Puf3 specifically binds to two CNUGUANAUA elements within the *COX17* mRNA 3′ UTR, leading to rapid deadenylation and subsequent degradation of the transcript.[164,165] This is achieved by recruiting the Ccr4-Not complex to the *COX17* mRNA through direct interaction with Puf3 (Figure 6**B**).[166] In etha-

nol growth conditions, the stability of *COX17* mRNA remains unchanged in comparison to *puf*3Δ strains, indicating that Puf3 mediates its degradation specifically under glucose conditions.[167] On the other hand, Puf3 also functionally stores mitochondrial mRNAs during fermentation by forming membraneless organelles.[13] When the carbon source is switched from glucose to ethanol, Puf3 undergoes phosphorylation, leading to the dissolution of the membraneless organelles and release of the mRNAs for rapid translation (Figure 6**B**), with the aid of the Pbp1 protein (poly(A)-binding protein) that Puf3 interacts with.[168] Although the study revealing the existence of Puf3 condensates does not explicitly state whether the membraneless organelles formed by Puf3 are located at the outer mitochondrial membrane, based on Puf3′s mitochondrial localization being unaffected by metabolic changes, it is reasonable to speculate that these structures are in proximity to the mitochondria.[169]

- *Regulation of gene expression*

a. Meeting demand

Studies on elements controlling mRNA localization to the mitochondria have indicated that mitochondrial targeting regulates protein expression levels, both in a positive way and to limit mitochondrial protein overexpression. It was shown for instance for *TIM50* that when the pausing was modulated and altered the efficiency of mitochondrial targeting (see above), this led to corresponding changes in *TIM50* expression, better localization of the mRNA at the mitochondrion resulted in better expression, and vice-versa.[154] Similarly, improving mRNA localization to the mitochondria by increasing relative mitochondrial volume enhanced overall protein production.[154,170] Moreover, as mentioned above, Puf3 is localized at the mitochondrion and is needed for sufficient expression of mitochondrial proteins during respiration.[169] While it has not been demonstrated that this is directly related to mitochondrial localization of the mRNA via Puf3, this seems a consistent explanation.

b. Avoiding overexpression and proteotoxicity

Nuclear-encoded mitochondrial precursors must be produced constantly to allow cells to adapt rapidly to increased demands for mitochondrial function. Hence under low mitochondrial demand, mitochondrial precursors are produced in excess and quality control systems maintain the cellular protein balance.[171,172] As mentioned above, ribosome pausing plays a role for targeting of mitochondrial protein-encoding mRNAs to the mitochondria. Thus, not surprisingly, the RQC pathway presented above that addresses excessive ribosome pauses during translation is relevant to regulate expression of nuclear-encoded mitochondrial proteins. The RQC mechanism will depend on whether the nascent protein chain is already within the mitochondrial import tunnel or not. If the nascent protein chain is not yet engaged within the mitochondrial import tunnel, the cell activates the RQC as described above. Otherwise, Vms1 at the mitochondrial outer membrane will inhibit Rqc2, preventing addition of a carboxy-terminal alanine and threonine tail through a noncanonical elongation reaction (CAT-tailing) to the stalled nascent chain. This will facilitate nascent chain import into the mitochondrial matrix, avoiding its aggregation and proteotoxic stress and allowing its degradation[173] **(**Figure 6**C).**

Recently, an integrated quality control system called Mito-ENCay has been described in yeast. This system was identified by defining how cells deal with overexpression of a nuclear encoded mitochondrial mRNA, *MMF1*, encoding a matrix protein, during growth of cells in glucose under low mitochondrial function demand.[84] Mito-ENCay involves various key players, including mitochondrial outer membrane proteins, chaperones, the Ccr4-Not complex, and the cytosolic disaggregase Hsp104, as well as components of RQC, NGD and autophagy. Deletion of any of these proteins or protein components increased the overexpression of *MMF1* mRNA and protein, as did reduction of ribosome pausing or reduced targeting of the mRNA to mitochondria.[84] In turn, ribosome pausing and regulation of overexpression was dependent upon on the optimality of the codon at the ribosome pause site as well as of Not4 of the Ccr4-Not complex. Moreover, limitation of overexpression required ubiquitination of Not4 targets such as NAC, the Rli1 ATPase and the ribosomal protein Rps7A.

A localized quality control at the mitochondrion involving similar factors seems to be conserved in flies and human. It was shown that mitochondrial mRNA co-translation quality control intersects with damaged mitochondria turnover, regulated by the PINK1 kinase. When damage occurs, the translation of the mRNA encoding the respiratory complex I30 kDa subunit (C-I30) stalls at the mitochondrion, recruiting quality control factors, PELO (Dom34 in yeast), ABCE1 (Rli1 in yeast), and NOT4, to the mRNA-ribonucleoprotein complex on the mitochondrial outer membrane. NOT4-mediated ABCE1 ubiquitination generates a poly-ubiquitin signal, attracting mitophagy receptors and selectively degrading damaged mitochondria.[172]

The importance of mRNA localization in temporally and spatially regulating gene expression was highlighted in a recent study revealing that in *Caenorhabditis elegans*, components responsible for mRNA 5′-3′ degradation and the Ccr4-Not deadenylase have both physical and functional associations with mitochondria, forming distinct foci. Specifically, foci of Ccr4, a subunit of the Ccr4-Not complex, function as mRNA storage foci whereas components for mRNA 5′-3′ degradation, like

DcpP1/2 and Xrn1, form foci serving for mRNA decay. Maintaining the balance between storage and decay foci is crucial for stress resistance and aging[11] (Figure 6**B**).

- *Protein dual localization*

Around a quarter of the mitochondrial proteome is suggested to be dual-localized, based on a combination of genome-wide screens and bioinformatic analyses.[174] The dual localization can be achieved through different mechanisms involving either one or two translation products. In the latter case, genes generate two translation products, one containing the MTS and the other lacking the MTS. These two translation products can arise at the level of DNA (involving two or more genes), mRNA (through transcription initiation or splicing), or translation initiation.[175] The dual-targeting mechanism allows a single protein to be localized to multiple organelles. This can be achieved through diverse mechanisms, in addition to proteins in two forms bearing or not the MTS, such as an ambiguous targeting sequence recognized by multiple organelles, multiple targeting sequences, changes in targeting sequence accessibility, or reverse translocation.[175,176] The yeast enzyme fumarase (Fum) has been extensively studied as an example of reverse translocation, where all Fum is initially targeted to mitochondria, where some of Fum undergoes protein folding and N-terminal signal peptide cleavage by mitochondrial processing peptidase (MPP) in the mitochondrial matrix, and then a fraction of Fum returns to the cytosol (Figure 6**D)**. The distribution of Fum is influenced by protein folding and the accessibility to molecular chaperones.[177–179] Importantly, *in vivo* and *in vitro* experiments consistently show that Fum enters mitochondria exclusively via co-translational import.[178,180] Fum's dual localization leads to diverse functions, with mitochondrial Fum participating in the TCA cycle, and cytosolic fumarase involved in the urea cycle and amino acid metabolism.[181,182] Recent discoveries reveal a novel role for cytosolic Fum in the cellular response to DNA double-strand breaks, as the enzyme translocates from the cytosol to the nucleus, actively participating in the DNA damage response through its enzymatic activity.[183]

Peroxisome associated mRNAs

Peroxisomes are membrane-enclosed organelles composed of both membrane proteins and matrix proteins. They are involved in the metabolism of reactive oxygen species (ROS), fatty acid oxidation, and cholesterol synthesis. The synthesis and assembly of peroxisomes is currently still subject of controversy. One model suggests *de novo* synthesis from the ER, while another proposes their formation through peroxisome fusion and fission.[184,185] Nevertheless, it is generally accepted that newly synthesized matrix proteins are post-translationally imported. However, the mechanisms for transporting membrane proteins to peroxisomes remain disputed. This controversy is partly attributed to certain peroxisomal membrane proteins lacking targeting signals. This raises questions about the proper localization of peroxisomal proteins facilitated by mRNA targeting. However, due to the small size of peroxisomes and their close association with the ER, the research into mRNA localization to peroxisomes is not always easy to interpret.

A comprehensive study employing single-molecule imaging and biochemical purification was conducted in yeast.[7] This study revealed distinct intracellular localization patterns for over 40 endogenously expressed mRNAs encoding peroxisomal proteins. For instance, mRNAs encoding Pex1, 5, 8 and 11 to 15 displayed high levels of co-localization with peroxisomes, whereas mRNAs encoding Pex6, 10, and 27 to 29 exhibited lower levels of co-localization. Other mRNAs like the ones encoding Pex2, 7, 17, 18, 22, 30 and 32 did not display co-localization. A different study utilizing electron microscopy coupled with proximity-specific ribosome profiling to identify a set of mRNAs associated with peroxisomes, did not always align with previous findings. The variation in the mRNA content associated with peroxisomes may be attributed to the dynamic nature of mRNA localization. In this latter study, they identified a set of mRNAs associated with peroxisomes, and electron microscopy revealed a cluster of ribosomes in close proximity to peroxisomes. Furthermore, they showed that tethering peroxisomal mRNAs to the ER or mitochondria resulted in cell growth deficiency.[186]

It is important to note that due to the close association between peroxisomes and the ER, distinguishing whether mRNAs encoding peroxisomal proteins localize to the ER or peroxisomes remains challenging. Deep sequencing of ER-associated mRNAs indicated an enrichment of mRNAs encoding peroxisomal membrane proteins, while soluble peroxisomal protein-encoding mRNAs did not exhibit such enrichment.[89] However, the purity of the isolated ER was not definitively established in these experiments.

Existing models propose that peroxisomal membrane proteins are synthesized *de novo* on the ER with subsequent budding, further complicating the discrimination between ER and peroxisomal mRNA localization. To address these challenges, advancements in high-resolution microscopy or optimization of isolated peroxisome purity will be crucial.

Nuclear pore complexes associated mRNAs

The cell nucleus serves as the compartment for mRNA synthesis and maturation, and it is separated from the cytoplasm by the nuclear

envelope. The presence of nuclear pore complexes (NPCs) within the envelope enables the exchange of substances and information between the cytoplasm and the nucleus. NPCs consist of approximately 30 subunits called nucleoporins (Nups). The mechanism of assembly of those subunits remains unclear. The assembly of the NPC is a complex field of research that is still at its infancy. Despite the fact that NPC protein complexes consist of multiple subunits, their assembly does not appear to involve chaperone proteins. This suggests that the assembly of the complex occurs co-translationally, reducing the risk of accumulation of intermediate products. A recent study in yeast has provided evidence to support this hypothesis. Through a systematic screening approach, it was discovered that the nuclear basket-associated proteins Mlp1 and Mlp2 are in contact with most Nup-encoding mRNAs. *NUP1* and *NUP2* mRNAs in particular were identified as colocalized with NPCs by a mechanism that involves interactions between their nascent chain N-termini and nuclear transport receptors. This indicated that NPC sub-complexes can assemble during translation. Disrupting the translation process abolished the association between mRNAs and NPCs futher supporting the notion. Moreover, this study indicated that nuclear transport receptors (NTRs) play a crucial role in bridging the interaction between NPCs and mRNAs.[8] However, this study did not rule out the possibility of involvement of other chaperones or mRNA motifs in this process, which still requires further investigation.

As mRNAs are synthesized within the nucleus, methods such as differential centrifugation, which is commonly used to separate subcellular fractions for sequencing, are not suitable for studying global mRNA localization to the nuclear membrane. Moreover, the current resolution of fluorescence microscopy makes it challenging to precisely determine the distance between the mRNA and the nuclear membrane, further complicating the study of nuclear membrane-associated mRNAs.

Conclusion and Perspectives

The intricate mechanisms of mRNA localization and targeting within cells add an additional layer of complexity to gene expression regulation. While much attention has been directed toward deciphering how mRNA localization contributes to the proper localization of protein products, it is equally important to recognize its significant role in temporally and spatially controlled regulation of gene expression.

Several intriguing questions remain to be explored in this field. Clarifiying the subsets of mRNAs that do show specific localization across diverse cytoplasmic regions, coordination of movement for co-regulated transcripts, and modulation of localization in response to physiological stimuli, will require further studies. Examining the prevalence and consistency of these localization patterns across different cell types would also enrich our understanding. Moreover, the potential of utilizing these patterns for predicting and manipulating protein function holds promise for both basic research and practical applications.

Advancements in methodologies for high-resolution and multiplexed RNA detection have ushered us into a new area for unraveling the intricacies of mRNA localization. These tools offer the potential to reveal regulatory connections and functional implications with unprecedented precision. However, the analysis of complex distribution patterns and their interconnections requires the development of suitable analytical techniques. To better understand the impact of membrane-associated mRNA regulation, integration with existing methods for profiling RNA is essential. The combination of methods for studying localized RNA with those focused on RNA-based regulation has the potential to provide a holistic view of the intricate relationships between mRNA localization, RNA stability, translation kinetics, and cellular structures.

In the near future, as technology continues to evolve, we can anticipate breakthroughs in deciphering the fundamental principles governing membrane-associated mRNA regulation. These discoveries will not only expand our knowledge of cellular processes but also pave the way for applications in disease research, therapeutic interventions, and bioengineering. With a growing arsenal of advanced tools at our disposal, we are poised to uncover new layers of complexity in gene expression regulation, revealing the cellular rheostats that intricately fine-tune spatially controlled gene expression.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used ChatGpt in order to refine the English grammar and language in the manuscript. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

CRediT authorship contribution statement

Siyu Chen: Writing – review & editing, Writing – original draft, Conceptualization. **Martine A. Collart:** Writing – review & editing, Supervision, Funding acquisition.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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