Protein targeting to subcellular organelles via mRNA localization.

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Cells have complex membranous organelles for the compartmentalization and regulation of most intracellular processes. The biogenesis and maintenance of organelles requires newly synthesized proteins, each of which needs to go from the ribosome translating its mRNA to the correct membrane for insertion or translocation to an organellar subcompartment. Decades of research have revealed how proteins are targeted to the correct organelle and translocate across one or more organelle membranes. The paradigm examples involve interactions between a peptide sequence in the protein, localization factors, and membrane translocation machinery. Membrane translocation is either cotranslational or posttranslational depending on the protein and target organelle. Meanwhile, research of embryos, neurons, and mating-type switching in yeast revealed an alternative targeting mechanism in which an mRNA is localized and only then translated to synthesize the protein in the correct location. In these cases, the targeting information is encoded by cis-acting sequences in the mRNA which interact with localization machinery and, in many cases, molecular motors for transport on cytoskeletal filaments. Recently, evidence has been found for this "mRNA-based" mechanism in organelle protein targeting to endoplasmic reticulum, mitochondria, and the photosynthetic membranes within chloroplasts. Here we review known and potential roles of mRNA localization in protein targeting to and within organelles.

Keywords: mRNA localization, co-translational and post-translational import, signal recognition particle, mitochondria, chloroplast, endoplasmic reticulum,

1. Introduction

Eukaryotic protein synthesis is preceded by transcription and maturation of the mRNA in the nucleus prior to its export through the nuclear pore complex (NPC) to the cytoplasm [1-4]. In the early view of translation, it was assumed that proteins are synthesized at random locations in the cytosol and then targeted to the different organelles using information in the polypeptide sequence [5]. This would suggest a post-translational translocation mechanism in which translocation is not aided by localization of the mRNA and ribosome to the organelle membrane. In the early 1970s the signal recognition particle (SRP) was discovered and found to bind the signal peptide of the nascent polypeptide, arrest translation, and then direct the mRNA-ribosome-nascent chain complex to the Sec61 complex in the endoplasmic reticulum (ER) membrane for cotranslational translocation insertion or translocation [6-9]. Thus, because these components were sufficient to target proteins to the ER in in vitro reconstituted systems, the peptide signal was considered to be the only information involved in the proper targeting of proteins to the ER (ref).

Over the past decade, mRNAs and ribosome subunits were shown to localize to the ER membrane in the absence of translation and, hence the signal peptide and nascent chain. These result raised the possibility that proteins are targeted to the ER by the localization of the mRNAs encoding them. On the one hand the exclusive role of SRP in protein targeting to the ER is being challenged [10, 11]. At the same time, the ER-localization of mRNAs coding for cytoplasmic proteins raised the question whether mRNA targeting depends on the formation of mRNA-ribosome complexes in the cytoplasm. For mitochondria of lower and higher eukaryotes a cotranslational mode of protein translocation is discussed in parallel to the most prominent post-translational mode [12, 13]. In addition, most mRNAs encoding mitochondrial proteins were not found equally distributed in the cytoplasm but enriched in the vicinity of mitochondria [14-16]. Although a cotranslational model for protein translocation into the chloroplast or the peroxisome has not been proposed, mRNA localization in close vicinity of these organelles has been observed as well [17, 18]. This review highlights recent results relating to the localization of mRNAs encoding organelle

proteins and discusses them in the context of the potential roles in the mechanisms and regulation of protein targeting, with an emphasis on mitochondria and chloroplasts. For more in depth coverage of potential roles of mRNA localization to the ER, the reader is referred to excellent reviews [19, 20].

2. mRNA-based protein targeting

While the organelle targeting pathways were being dissected, a distinct protein targeting process was discovered by researchers of animal development, neuronal plasticity, and the regulation of mating-type switching in the budding yeast *Saccharomyces cerevisiae*. In this mode, a cis-acting sequence element in the mRNA specifies its localization, in an untranslated state, whereupon translation ensues to produce the protein in the proper location [21, 22]. In addition to targeting the protein, this mRNA-based targeting may also function to: i) exclude the protein from intracellular regions where it would be toxic, ii) circumvent the requirement for targeting mechanisms and complexes for the localization of proteins to the distinct compartments of the cell, iii) ensure rapid translational responses to changing abiotic or biotic conditions, iv) allow the regulation of the protein synthesis by cellular and extracellular stimuli that reflect demand for the product, v) provide economic benefits from not having to localize the many copies of a protein translated from a single localized mRNA, and iv) establish “privileged” translation sites that might be secluded from other regions under stress (cite http://www.ncbi.nlm.nih.gov/pubmed/16540694). Below, a brief overview of the components of mRNA-based localization is followed by reviews of emerging roles of this mechanism in organellar protein targeting.

Consistent with the importance of this mode of protein targeting, specific localization patterns of were observed for mRNAs in many organisms and cell types such as yeast [23, 24], *Xenopus* [25], Neurons [26, 27], *Drosophila* [28, 29] and plants [20, 30-32]. Interestingly, bacteria also localize mRNAs to distinct regions in the cell even though they do not have subcellular compartments [33]. mRNA localization in the embryo of *Drosophila melanogaster*

has many roles in pattern formation with approximately 70% of mRNAs being specifically localized [34].

In many cases, the mRNAs are transported as high molecular weight mRNPs in a translation-repressed state. The active transport throughout the cytoplasm occurs on the cytoskeleton by the molecular motor proteins (reviewed in detail by [35-37]). Alternative modes of mRNA localization involve local stabilization [38, 39] or the capture and tethering after passive diffusion [40, 41].

Localization is specified by a cis-acting sequence in the mRNA called a localization element (LE) or Zipcode [42]. Zipcodes range from only few nucleotides [43] to highly complex and redundant sequences of up to 1 kb [44]. They are most often located within the 3'UTR and in most cases sufficient for the localization of a reporter mRNA. Currently, many of the 3'UTR features leading to mRNA localization are known (and summarized in [45]), and were found by experiments using fluorescence microscopy [46] or cross-linking and immunoprecipitation (CLIP) [47]. RNA-binding proteins bind to these sequences and localize them by the various mechanisms reviewed above.

3. The mRNA association with the membranes of the nucleus and the ER

3.1 Translation dependent mRNA association

Localization of mRNAs to the ER was discovered decades ago [48]. The initial description was the discovery of the cotranslational translocation mechanism by the SRP, which recognizes the N-terminal signal sequence of the nascent chain. This leads to the association of the mRNA-ribosome-nascent chain complex to the Sec61 complex for contranslational translocation or membrane insertion [6-9]. In the recent years it became evident that not only the mRNA of secreted proteins, but mRNAs encoding cytosolic proteins are localized at the ER surface by RNA intrinsic signals [49]. Furthermore, specific mRNAs are associated with the ER surface for regulatory purposes [50].

The paradigm for protein targeting of proteins to the ER for secretion or the plasma membrane is the SRP pathway [6, 51-55]. The action of SRP is threefold: it recognizes the

signal sequence on the nascent polypeptide emerging from the ribosome, arrests translation, and then targets the ribosome-mRNA-nascent chain complex to the SEC complex for cotranslational membrane translocation or insertion. As a consequence, the mRNA associated with the ribosome is targeted to the ER surface by cis-acting sequence in the polypeptide. This model is largely based on now classic *in vitro* studies documenting that the signal sequence is both essential and sufficient for targeting of the of the ribosome to the ER surface [56]. Consequently this type of mRNA localization is translation-dependent and independent of cis-acting sequences in the mRNA itself.

3.2 Translation independent association of mRNAs encoding ER and secreted proteins

In recent years, evidence has emerged to indicate that the concept of an exclusive requirement on the signal sequence or SRP has to be modified. On the one hand, SRP deficiency in several eukaryotes did not cause lethality as expected if this factor is required for protein targeting to the ER because this process is essential for viability [57-59]. On the other hand, polypeptide independent association of the mRNA coding for proteins targeted to the ER was observed. For example, Pmp1p, a small plasma membrane localized protein in *S. cerevisiae*, is synthesized into the ER membrane and routed via the secretory pathway. However, its mRNA contains a 3' UTR localized motif which is involved in its association with the ER membrane [10]. The motif consists of an UG rich region and most likely forms a hairpin, the structure of which was envisioned to be relevant for the functionality of the motif. By sedimentation experiments it was shown that the 3' UTR fused to open reading frames coding for other proteins leads to a shift of their migration indicative of a membrane association [10]. It is presumed that this functions to localize the mRNA for the synthesis of Pmp1p at the ER-membrane for SRP-independent insertion into the ER membrane. Thus, for Pmp1p, and possibly other proteins less the minimum length of a nascent chain required for signal sequence based targeting (50 amino acid residues) [60], the sequences in the mRNA contributes to its ER targeting.

In recent studies in mammalian cells this concept was further extended [11, 61]. It was discovered that mRNAs coding for proteins that reside in the ER are associated with the ER in a ribosome independent manner, most likely in addition to the SRP-mediated association [11, 61, 62]. Ribosomes were also found to be associated with the ER independently of translation and a nascent peptide, suggesting that they are also localized by translation-independent mechanisms [60]. In the light of the observed association of mRNAs encoding cytosolic proteins (see below) it is suggested that this might reflect a global mechanism of the ER in the protein synthesis. Such a role may be particularly important for the synthesis of proteins required for cell division because it distributes the messenger into the daughter cell [11, 61]. Alternatively, the association of mRNAs encoding components of the chaperone network of the ER-lumen, e.g. Bip/Grp94p, might reflect a requirement for a rapid production of these proteins during the unfolded protein response. Therefore, association of certain mRNAs to the ER surface might be an integral component of the cellular signal transduction network. Nevertheless, the ribosome independent association of mRNAs imported by the cotranslational SRP pathway is revealing that many proteins are targeted to the secretory system by the concerted action of two pathways, one being mRNA-based and translation-independent and the other signal peptide-dependent and translation-dependent. One possibility is that the mRNA-based pathway localizes the mRNA and ribosomes to the membrane for the initiation of translation, where upon the SRP pathway takes over and carries out the docking steps to the Sec61 translocon.

3.3 ER association of mRNAs coding for non-organellar proteins

In addition to mRNAs of proteins residing in the lumen or engaging the secretory pathway, the mRNAs of cytosolic and nuclear proteins were found to be associated with the ER membrane as well. The discussion of the ER-association of mRNAs coding for cytosolic proteins was already initiated in the early 1980s. By the analysis of the mRNA composition of cytosolic and membrane bound polysomes, a substantial overlap between the two pools was observed [63, 64]. This observation was subsequently confirmed by results of microarray

analysis of the mRNA population associated with ER [61, 65-67]. It was proposed that the mRNAs of cytosolic proteins are recognized by ribosomes that have completed synthesis of ER-targeted proteins and induce the elongation-induced ribosome release [68], a possibility that was supported by results in *in vitro* experiments [69, 70]. At the same time, mRNAs of soluble proteins were found to be associated with the perinuclear surface [62]. A recent comparison of the mRNA content of the cytosolic and nuclear envelopes – most likely including the ER membrane – revealed an overlap of these pools as large as 9000 mRNAs in mammals [11]. At present it is discussed that the direct connection between nuclear envelope and the ER membrane might serve to deliver the messengers to the translation machinery, as membrane bound ribosomes are capable of translation initiation of mRNAs coding for cytosolic proteins [69]. In addition, the association of mRNAs with the ER membrane may be a mechanism for mRNA partitioning to daughter cells during cell division.

The *ASH1* mRNA in *S. cerevisiae* is a particularly well-understood example of how mRNA localization to ER can target a protein to a location outside the secretory system, in this case to the budding daughter cell. ASH1p is required specifically in the daughter cell to repress transcription of the gene encoding the HO nuclease, which initiates mating-type switching in the mother cell. During budding, the equivalent to cytokinesis in *S. cerevisiae*, the entire *ASH1* mRNA pool is trafficked from the mother cell to the daughter cell. This was shown to involved *ASH1* mRNA association with ER by fluorescence microscopy and cellular subfractionation [72, 73]. Only in the daughter cell is the mRNA translated to produce ASH1p where it localizes via the cytoplasm to the nucleus.

Mechanisms involved in this mRNA-based targeting of ASHp have been demonstrated. *ASH1* mRNA localization involves a complex interplay between an RNA-Zipcode in its sequence, a variety of RNA-binding proteins, molecular motors, the actin cytoskeleton, and the cortical ER of the budding daughter cell. (reviewed by http://www.ncbi.nlm.nih.gov/pubmed/18262421). The localization to the budding daughter cell is dependent on Puf6p, which binds to the *ASH1* mRNA 3' UTR and represses translation [74]. The ER association of the mRNA is dependent on She2p [75], which

contains a basic helical hairpin motif [76] and associates with the mRNA when it is still in the nucleus [77]. In addition to directing nuclear export of the *ASH1* mRNA [78, 79], once in the cytoplasm She2p engages a complex with Myo4p, a type V myosin [80-82]. Recruitment of She1p and She3p then partition this complex containing the ASH1 mRNA to the ER of the budding daughter cell via an as yet unknown mechanism [73, 83] [84].

The RNA-Zipcodes in the *ASH1* mRNA mediate the interactions with She2p and the ER membrane [85]. Four of such elements were identified in the ORF with one extending into the 3' UTR. These functionally redundant elements are annotated as E1, E2A, E2B, and E3, [24]. They appear to be conserved at an RNA structural level by exhibiting a similar stem-loop structure, but they lack sequence similarity [86, 87] other than a conserved CGA triplet in one loop and a critically spaced cytosine residue [88]. A similar structure containing the triplet and the conserved cytosine were also discovered in other mRNAs that are localized to the budding daughter cell [88], thereby supporting the general role importance of this RNA-Zipcode for ER-membrane localization via She2p.

In another example of mRNA association with ER for protein targeting involves mammalian Dia1p, a cytosolic actin nucleation factor. Its mRNA is associated with the perinuclear ER membrane in a "RNA-Zipcode independent mechanism" [89, 90]. In this case, the association of the mRNA to the ER is thought to be mediated by the interaction of nascent Dia1p and the ER localized Rho-GTPase [89, 90]. Thus, it would parallel the conventional mode of mRNA association by ribosome SEC interaction with the only distinction that the interaction partners are Dia1p and Rho, rather than SRP and SRP-receptor. The functional reason for this association, however, remains unknown.

3.4 ER association of mRNAs for the regulation of the unfolded protein response

A distinct mechanism is found for Xbp1/Hac1/bZIP60 (human/yeast/plants), which is a substrate of Ire1p and activated in the unfolded protein response (UPR) [91, 92]. For the yeast factor Hac1p it was described that a sequence element which is present in the 3' UTR is essential for targeting to the ER surface [93]. This targeting, however, occurs only during

the UPR with only a little fraction bound to the surface beforehand [66, 93]. In the cytosol the translation is arrested by long-range base pairing of the *HAC1* mRNA [94], which is disrupted by Ire1p dependent splicing of the mRNA [95].

For the human homologue of Hac1p, Xbp1p, it was observed that the mRNA is already attached to the ER membrane prior to the induction of the unfolded protein response. At this stage the intron is not spliced out and thus, the mRNA contains a frame shift with respect to the mRNA encoding the active form. Its translation leads to the synthesis of a C-terminal truncated Xbp1p variant with a hydrophobic amino acid stretch annotated as HR2 [96]. This hydrophobic region interacts with the membrane and causes translational arrest [96, 97]. Both, the association of the hydrophobic segment with the membrane and the translational arrest are prerequisites for the association of the ribosome bound mRNA to the ER, thus in close vicinity of the Ire1 machinery responding to the UPR.

A mode rather comparable to the human system than to the yeast system was described for the plant protein bZIP60, where under normal conditions the non-spliced form is translated and at least associated with (if not inserted into) the ER-membrane by the existence of a C-terminal hydrophobic domain [98, 99]. Initially it was discussed that the transition from membrane bound to non-membrane bound form of bZIP60 is induced by a proteolytic event [98], but further analysis revealed that the transition is modulated at the mRNA level by an Ire1 dependent processing of the according mRNA [99]. Whether the localization of the mRNA occurs before cleavage is not known, but it might parallel the mechanism observed for human *XBP1* RNA as described above.

3.5 The different modes of mRNA association with the ER surface – a summary

To summarize, mRNA are localized to the ER in the classical translation-dependent SRP pathway and a translation-independent mRNA-based pathway. The distribution of mRNAs of classical cotranslational import substrates most likely depends only on the polypeptide (Figure 1a). However, not only for the cotranslational translocation, also for the assembly of complexes or for the regulation of mRNA splicing as regulatory event an exclusive

polypeptide based mRNA association with the ER-membrane can be found. For small proteins, which engage the secretory pathway but for which the polypeptide chain is too short to emerge from the ribosomal exit tunnel for recognition by the SRP and alternative mode of targeting has evolved. Here, the signal for targeting appears to be transferred to the mRNA as a ZIP-code in the 3' UTR (Figure 1b). At the same time it appears that ribosome independent association exists, on the one hand as regulatory mechanism for ER-localized chaperones, on the other hand for the association of mRNAs coding for cytosolic proteins involved in the definition of cell polarity or localized to distinct cellular positions during division (Figure 1c).

4. mRNA localization to mitochondria

Proteins are imported from the cytoplasm into chloroplasts, mitochondria, and peroxisomes by the recognition of a transit peptide sequence, generally located at their N-terminus, by the import machinery. As for the SRP pathway, the transit peptide is removed by proteolytic cleavage following import. While import in these cases is believed to occur after the completion of translation, recent results clearly indicate an additional cotranslational translocation mechanism into mitochondria. This mechanism is indispensable for several proteins that if fully translated in the cytosol would aggregate to an import-incompetent form and thus require the coupling of translation and import [13, 100-102]. Furthermore, mRNAs encoding mitochondrial proteins are enriched at the mitochondrial surface and specific RNA-binding proteins localize to mitochondria to recruit these mRNAs [14-16, 103]. Additional evidence exists for an important role in mRNA localization to mitochondria during conditions that require high rates of protein import [104].

4.1 mRNA localization to mitochondria – cotranslational vs. post-translational import

While a few proteins are encoded by mitochondrial genomes and synthesized by 70S bacterial-like ribosomes within the organelle, the vast majority of mitochondrial proteins are encoded by the nuclear genome and synthesized by 80S cytoplasmic ribosomes. Most of the

proteins designated for their localization in mitochondria contain a transit peptide: an amino acid sequence at the N-terminus that serves as a posttranslational targeting signal [99]. After successful translocation into the mitochondria through the TOM/TIM complex (Figure 2a) this targeting sequence is cleaved off by the mitochondrial processing peptidase (MPP) to yield the mature protein. Thus, many reports of post-translational protein import into mitochondria in *in vitro* import assays and *in vivo* led to the assumption that the majority of mitochondrial proteins utilize this pathway [105]. However, several studies indicate that in some special cases obligate cotranslational mitochondrial protein import exists in which the mRNAs together with cytoplasmic (specialized) polysomes can be found in close vicinity to the outer membrane of mitochondria [13, 106-110]. As discussed in the next section, recent studies reveal a more prominent role for localized mRNAs at the surface of mitochondria.

4.2. Global analysis of mRNAs bound to the mitochondrial surface

In the early 1970s, several groups challenged the hypothesis of ribosomes or polysomes bound to the mitochondrial membrane in several organisms. Several reports showed that somehow specified ribosomes (different KCl stability than free cytoplasmic ribosomes) are associated with the mitochondria in cell fractionation experiments [106, 108, 109]. These ribosomes are of cytoplasmic nature (80S) and could be linked to the mitochondria via the nascent peptide chain engaged in translocation by the TOM-complex in the outer membrane of the organelle. Already in these early studies, specific binding sites for ribosomes on the mitochondrial surface were proposed because EDTA-washed (“stripped”) mitochondria are able to interact with isolated 80S ribosomes in a Mg^{2+} dependent manner [106, 108, 109]. Using EM of yeast spheroplasts, polysomes were observed at the vicinity of mitochondria, the ER and nuclear membrane, but not at the plasma membrane or vacuole [107].

In 1980, the fate and composition of polypeptides synthesized from mitochondria-bound polysomes was analyzed using specific enzymatic assays for mitochondrial and cytoplasmic proteins [111]. A higher enzymatic activity of the mitochondrial cytochrome c oxidase and isocitrate dehydrogenase was observed when proteins were synthesized from

mitochondria bound polysomes in comparison to cytosolic polysomes [111]. This result indicated that mitochondrial mRNAs are enriched in organelle associated polysomes. Similarly, the mRNAs for α-, β- and γ-subunits of the F1 ATPase are also enriched on polysomes bound to mitochondria [111].

This observation was subsequently confirmed and extended to other mitochondrial localized polypeptides [112]. By hybridization of mRNAs (cDNA) from mitochondria-bound and free polysomes from yeast to DNA microarrays a so called MLR value (mitochondrial localization of mRNA) for each gene was calculated [14]. About half of the mRNAs encoding for known or putative mitochondrial proteins had high MLR values suggesting their mitochondrial association. Interestingly, most of the mRNAs with high MLR value were of bacterial origin (e.g. fumarase, malate dehydrogenase). These authors proposed that cotranslational import may predominate for proteins that were encoded by the genes of the ancestral alpha-proteobacterial genome that have transferred to the nucleus during evolution [14].

The observations for yeast were subsequently extended to mammals. A tight binding of ribosomes to rat liver mitochondria was shown, which is thought to be regulated by GDP/GTP and the transit-peptide in the nascent chain of a protein designated for mitochondria [113]. Further support for obligate cotranslational import into mammalian mitochondria was provided by the results of *in vitro* import assays of two proteins being synthesized in the presence of import-competent mitochondria from rat liver. It was found that the import of one protein (Adenylate Kinase 2) required translation, because it was inhibited by treatment with cycloheximide, an 80S ribosome inhibitor. Import of Adenylate Kinase 3 was not similarly inhibited, arguing that the translation-dependence was not an artificial effect of cycloheximide [102]. These findings suggested that different modes of translocation exist. Indeed, some evidence suggests a mitochondrial association of mammalian ribosomes which requires Mg^{2+} ions and surface proteins, presumed to be the localization factors [114]. Consistently, cotranslational import for several mitochondrial and of the artificial fusion of a transit-peptide to DHFR was suggested based on *in vitro* experiments

[101]. For the artificial precursor protein it was estimated that about 70% of the precursor needs to be imported cotranslational [101] suggesting both cotranslational and post-translational import function in parallel.

It was further confirmed that the mRNA targeting mechanism involving the 3' UTR is evolutionary conserved. The human OXA1 protein can rescue the yeast deletion strain of Oxa1p. [115]. Interestingly, the 3' UTR of the *HsOXA1* mRNA is essential for localization to yeast mitochondria and the first 60 amino acids containing the mitochondrial targeting sequence (mts) can be omitted [115].

4.3. Factors involved in the mRNA localization to the mitochondrial membrane

Six Puf (Pumilio-Fem-3 binding factor) proteins exist in yeast, which contain several repeats of a RNA-binding domain called Pumilio. Puf6p, for example, could be co-purified with She2p-mRNPs containing *ASH1* mRNA that is localized to the bud tip in yeast in a translational repressed state [24, 74, 79, 116]. *In vitro* Puf6p binds to the 3' UTR of Ash1 mRNA and thereby represses its translation during the transport in the cytoplasm [74]. For Puf3p a function in localizing transcripts to the mitochondrial surface was suggested [16, 103, 117]. Almost exclusively nuclear genes coding mitochondrial proteins were found to be associated with Puf3p in a systematic approach to identify targets for the Puf-proteins 1-5 [103]. Further, the consensus RNA motif to which Puf3p binds can be found in the 3' UTR of 270-300 nuclear genes encoding for mitochondrial proteins [118, 119]. Consistent with a function in mRNA localization to mitochondria, Puf3p co-localizes with mitochondria at the periphery of the outer membrane [120]. The protein is involved in the regulation of mitochondrial biogenesis and motility in budding yeast as the puf3Δ-strain showed an abnormal motility and altered morphology of mitochondria [120]. The microarray based analysis of mitochondria-bound mRNAs in the puf3Δ-strain [16] uncovered two classes of mitochondria bound mRNAs: CLASS I mRNAs depend upon Puf3p as they are mislocalized in the deletion strain and CLASS II mRNAs are Puf3p independent (Figure 2b). This classification suggests that nearly 40% of the mRNAs were associated with the mitochondrial

surface in a Puf3p-independent manner. Still, both classes have in common a translation requirement of their association to the mitochondria, which is somewhat sensitive to the inhibition of translation by cycloheximide treatment. .

Puf3p is even more interesting as it usually causes deadenylation and repression of some of its mRNA targets [117, 121]. Even though the repressive role of Puf3p is contradictory, two possibilities were suggested by a recent review on the Puf-proteins: i) two pools of Puf3p exist (a mitochondrial and a non-mitochondrial pool) and the non-mitochondrial localized Puf3p acts as a repressor and ii) translational repression at the mitochondrial membrane may slow down translation for efficient cotranslational import [121].

mRNA localization to mitochondria was further investigated in puf3Δ- or tom20Δ-strains [15] utilizing a m-Tag gene-tagging system, which uses several MS2 binding sites inserted in the mRNA sequence [46, 122]. Additionally, the yeast strains express the MS2 coat protein fused to several copies of GFP to amplify the signal. Although the background overlay fluorescence is approximately 24% it could be demonstrated that the mRNAs encoding *ATP2* and *OXA1* localize to mitochondria strongly dependent on Puf3p and their 3' UTR. Interestingly, a functional TOM-complex is additionally required for correct mRNA localization. However, Puf3p levels rise in the tom20Δ-strain as the cause of mislocalization of the protein. Thus, the influence of the TOM complex on mRNA localization might be a pleiotrop result of Puf3p mistargeting [104]. However, it was shown that deletions of both Tom20p and Puf3p together are synthetic lethal for growth on a respiratory carbon source (e.g. glycerol) suggesting that these proteins cooperate in mitochondrial protein import at least for a subset of mRNAs [104]. Nevertheless, this explanation is not valid, at least not for all mislocalized mRNAs in the tom20Δ-strain, because the effects of the TOM-proteins Tom7p or Tom20p were specific for a subset of mRNAs and not globally for all Puf3p dependent mitochondrial associated messengers [15, 104]. Furthermore, Tom20p indirectly interacts with a specific subset of mRNAs which lack the 3' UTR consensus motif for Puf3p [104]. In conclusion, yeast cells possess an mRNA mitochondrial association mode that

involves Tom20p and the translated MTS and Puf3p assists in the association of some mRNAs through interaction with their 3' UTRs.

4.4 Fumarase and ATP2 – two models for the analysis of alternative targeting modes

For certain native and artificial precursors proteins the need for cotranslational import - and thus mitochondria localized mRNAs – was demonstrated to function to prevent their aggregation of prior to import [12, 13, 100, 123]. This concept was for example based on the analysis of CoxIVp fused to dihydrofolatereductase as an artificial passenger. This precursor was not sufficiently imported when the precursor protein was fully synthesized *in vitro* and then subjected to mitochondria. However, the presence of isolated mitochondria in *in vitro* translation reactions led to highly efficient import [123]. This observation suggested a coupled translation and translocation at least for this protein fusion [123]. The idea of a cotranslational import was further supported by the finding that the inhibition of DHFR unfolding by antifolate methotrexate had no effect on the *in vitro* import rate when the coupled system was used. Similarly antifolate methotrexate had no effect on import when added to cells [100]. Considering that proteins must be unfolded as they pass through the import machinery, these results support cotranslational import.

The relevance of the discovered system was demonstrated for the precursor of fumarase, a protein which, in the mature form, is localized in the cytoplasm and mitochondria [124]. The enzyme is partially imported into mitochondria, processed and retrograde exported back into the cytoplasm yielding two different localized proteins that cannot be distinguished by post-translational modifications or protein size [125]. Forced cytoplasmic accumulation and folding of fumarase led to inhibited import into mitochondria, which is consistent with the requirement for coupled translation and translocation (Figure 2a). By using fumarase constructs with inserted TEV cleavage sites, it could be demonstrated that under normal conditions TEV cleavage did not occur in the cytosol suggesting a fast rate of coupled protein import into mitochondria [13]. However, slowed down translocation in a *tom40*ts mutant results in cytosolic cleavage of fumarase which supports the fast kinetics of

protein import [13]. This strongly suggests that the coupled translocation requires the TOM complex.

Further candidates for a coupled translation and translocation are Atp2p and Atm1p,. The *ATP2* mRNA encodes the ß-subunit of the *F1-ATP* synthase, also called respiratory chain complex V [126]. *ATM1* encodes an ABC transporter of the inner mitochondrial membrane [127]. In the genome-wide studies it was shown that the *ATP2* mRNA as well as the *ATM1* mRNA exclusively localize to mitochondrion-bound polysomes and both belong to the Puf3p-independent class II mRNAs [14, 16]. These observations were confirmed by northern blotting of mitochondrion bound and free mRNA populations, revealing that both mRNAs are bound by mitochondria. [128]. Contradictory, in the latest study, the *ATP2* mRNA localization to mitochondria was clearly dependent on Puf3p even though this mRNA does not bear the Puf3p consensus binding site in its 3' UTR [15]. One possibility is that mRNAs localized to mitochondria are transported as large mRNPs with diverse mRNAs in it but that issue was not further addressed.

Recent studies analyzed the sequence elements required for the asymmetric localization of the *ATP2* mRNA to mitochondria and the consequence of sequence alterations for protein function [126, 129, 130]. First, it was shown that the mitochondrial targeting sequence (mts) or a sequence within the 3' UTR of the *ATP2* mRNA were sufficient to target the mRNA to mitochondria (Figure 2c) [130]. A 3' UTR swap experiment revealed that the *ATP2* 3' UTR is necessary for mitochondrion-association and import of the protein product *in vivo*. [126]. A similar result was obtained for the *ATM1* mRNA which associates in a translation-independent manner with the mitochondrial surface and in which the targeting Zipcode is also redundant (3' UTR and mts) [128].

Additionally to the importance of the 3' UTR it was discussed that the first steps of mitochondrial import precede the anchoring of mRNA to mitochondria (Figure 2c) [129]. However, this does not explain how the 3' UTR of *ATP2* mRNA is sufficient to target a reporter gene to the mitochondrial surface, which suggests that mRNA localization occurs independent of import as well and both the nascent chain and the 3' UTR work in parallel or

each on different sets of mRNAs [126, 131, 132]. Furthermore, recently it was shown that cytosolic chaperones that work downstream of NAC are involved in mRNA targeting [133].

4.5 Summary on the biological role for mitochondrial localized mRNAs and coupled translation-translocation

In the last decade it was established that about half of the cytoplasmic mRNAs encoding mitochondrial proteins are located to the vicinity of mitochondria. As consequence one can suggest that translation-coupled translocation acts in conjunction with post-translational protein import. For some proteins it was shown to be indispensable that they are translocated into the mitochondria prior to a completed translation in the cytosol [13]. For these proteins the biological importance of localized mRNAs is to prevent aggregation of highly hydrophobic proteins in the cytoplasm. Although there is an ongoing discussion about cytosolic HSPs that keep precursor proteins in an unfolded-import competent state [134, 135], one might suggest that this cannot ensure post-translational translocation in every case. This proposal considers that one can differentiate between mRNAs that are translation-dependent localized to the mitochondria to prevent protein aggregation and mRNAs that are associated with the organellar surface dependent on RNA-binding factors like Puf3p (or other unknown factors) prior to translation [16].

Mitochondria contain macromolecular complexes in the two membranes and in the matrix whose assembly involves many accessory proteins. For example, the mRNA of all 16 proteins of the succinate:quinoneoxidoreductase (SQR) were found to be associated with the mitochondrial membrane [136]. The same holds true for the majority of proteins and assembly factors of the ATP synthase complex, the COX complex (RC4) and the bc1 complex (RC3). Remarkably, the mRNA of at least one component of each of the mitochondrial translocation machineries was found to associate with the mitochondrial surface as well [136]. Interestingly, most of the proteins encoded by mitochondria-associated mRNAs are from genes of bacterial origin and encode for the highly hydrophobic core subunits of macromolecular complexes. In this context it is worth mentioning that the inner

membrane protein Oxa1p binds mitochondrial ribosomes and facilitates cotranslational membrane insertion of the hydrophobic Cox2p protein [137] and that the translational activator proteins binding to a mRNA region of *COX1-3* are physical connected at the inner membrane and are involved in facilitation of cotranslational core COX complex assembly [138, 139]. The results suggest a role for mitochondrial mRNA localization for the regulation of protein translocation and proper complex assembly.

5. mRNA association with membranes of peroxisomes and chloroplasts – a functional importance for protein translocation?

Protein targeting to chloroplasts or peroxisomes has long been believed to be entirely posttranslational [17, 18]. However, there is evidence for localization of mRNAs to peroxisomes and within chloroplasts.

5.1 mRNA association with peroxisomes

Peroxisomes are single-membrane-bound organelles lacking DNA and ribosomes [140, 141]. All integral and membrane embedded peroxisomal proteins are encoded by the nuclear genome and translated by 80S cytoplasmic ribosomes. The current model holds that protein translocation into peroxisomes occurs post-translationally by the targeting of the proteins containing peroxisomal targeting signals [142]. Common peroxisomal targeting signals are a C-terminal tripeptide (usually Ser-Lys-Leu (SKL); PTS1), a N-terminal peptide sequence (PTS2), and the mPT, a signal of peroxisomal membrane proteins [143]. The peroxisomal import machinery, called the “importomer”, has a large and highly dynamic pore wide enough for the import of gold particles (Ø ~9nm) coated with PTS1 [144, 145]. Thus, it is assumed that proteins targeted to peroxisomes probably do not require to be unfolded and therefore the abovementioned aggregation problem of hydrophobic mitochondrial proteins may not apply.

Nevertheless, cotranslational protein import might be envisioned as additional mode for processing of peroxisomal proteins. Firstly peroxisomes can assemble spontaneously

starting with Pex3 arising from the ER [146, 147], Secondly, the majority of peroxisomal membrane proteins traffic through the ER [148, 149]. Thirdly, mutations of genes involved in secretion like *SRP54* affect the biogenesis of peroxisomes [150]. However, these pathways are rather linked to the cotranslational insertion of proteins into the ER-membrane. However, in *S. cerevisiae*, mRNAs encoding peroxisomal membrane proteins (mPP) were seen to co-localize with peroxisomes *in vivo* which have been induced by oleate treatment and this association might again be mediated by the 3' UTR [18]. Furthermore, for some peroxisomal localized mRNAs the results were confirmed by cellular subfractionation. The function of these mRNA associations with peroxisomes is unclear and additional work is required to address the questions raised by this study. Nevertheless, at least two mRNAs coding for mPPs (PEX14 and PEX22) are targets of Puf5p [103] and loss of Puf5p results in less co-localization of PEX14 mRNA with peroxisomes [18]. However, the cellular localization of Puf5p remains to be established and it remains to be explored whether this RNA-binding protein may have a similar role to that of Puf3p in mRNA localization to mitochondria (Section 4.3).

5.2 *Are mRNAs encoding chloroplast proteins localized for translation and targeting of their polypeptide products?*

In plants and green algae, approximately 1,300 proteins are imported into chloroplasts to function in photosynthesis, the expression of the chloroplast genome, and several biosynthetic pathways [151]. A longstanding view holds that these proteins are fully synthesized at random cytoplasmic locations and then directed by N-terminal transit peptides to the import apparatus in the chloroplast envelope for posttranslational import. This post-translational import model is based on the ability of isolated chloroplasts to import *in vitro*-synthesized proteins and the apparent absence of ribosome in the immediate vicinity of chloroplasts, as seen in EM images [152, 153]. Protein import occurs via translocon complexes in the inner and outer membranes of the chloroplast envelope which have been

dissected over the past two decades primarily with experiments carried out *in vitro* or *in organelle* and with few *in situ* and *in vivo* experiments [154-158].

Little attention has been given to the question of whether or not the mRNAs encoding chloroplast proteins are localized for translation and import of their polypeptide products. However, the possibly that mRNA localization plays a role in chloroplast protein targeting was raised by two findings. First, the mRNA encoding a chloroplast protein and 80S cytosolic ribosomes were seen to colocalize at specific regions of the chloroplast perimeter in the green alga *Chlamydomonas reinhardtii* by fluorescence *in situ* hybridization (FISH) and immunofluorescence (IF) staining [17]. The polypeptide encoded by this mRNA, a light-harvesting complex II subunit, is known to be imported across the chloroplast envelope via the TIC and TOC translocon complexes, suggesting that protein synthesis and import by this pathway are spatially coordinated at specific regions of the chloroplast envelope. The second suggestion of localized translation arose with evidence of an alternative pathway by which certain proteins are routed to chloroplasts via the ER and Golgi. This is based on the finding of glycosylated proteins in the chloroplast of rice and Arabidopsis and the fact that glycosylation occurs only in the Golgi [159, 160]. If a branch of the secretory system pathway to the chloroplast does exist, it might involve the localization of translation cis-acting Zipcode sequences in the RNAs (Section 3) because only 0.6% of chloroplast proteins are have the predictable signal peptide sequence, recognized by SRP to cotranslational localization to the ER [151]. Other evidence for specialized domains of the ER associated with chloroplastsalso supports the existence of such alternative import pathway. For example, the extreme example is “chloroplast ER” with bound ribosomes that was described decades ago in EM studies in several groups of algae [161] and more recently in vascular plants [159, 162-165].. The algae with extensive chloroplast ER, for example*, Ochromonas danica,* may provide ideal model systems for the exploration of protein routing to chloroplasts via an ER pathway . The cytological organization of chloroplast protein synthesis and import is an area ripe for discovery.

5.3 Evidence for mRNA-based protein targeting within chloroplasts.

Chloroplast genomes encode some 100-200 proteins of the photosynthetic apparatus and organellar gene expression system and a bacterial-like genetic system, reflecting their evolution from a cyanobacterial endosymbiont [166]. Many of these "chloroplast-encoded" proteins are targeted to thylakoids, a network of flattened membranous vesicles, where they function as subunits of the photosynthetic electron transport chain and the CF_1F_O-ATP synthase (reviewed by [167]).

Five lines of evidence support the existence of mRNA-based targeting of proteins encoded by chloroplast genomes. First, the signal sequence binding protein of the chloroplast's SRP pathway, cpSRP54, is dispensable for the targeting of at least some photosynthesis proteins to thylakoid membranes in Arabidopsis [195] [194, 196-198]. This result demonstrates the existence of at least one other targeting mechanism, possibly, but not necessarily, one involving mRNA localization to thylakoid membranes (reviewed by [199, 200]. Second, while most translating ribosomes in chloroplasts are membrane-bound, approximately 50% of them are held only by electrostatic interactions, i.e. independently of a nascent chain in Chlamydomonas [171, 173]. This result suggests that polysomes are tethered to membranes by proteins that bind chloroplast polysomal mRNAs, ribosomes, or both, as in mRNA-based targeting to the ER (Section 3). Third, several membrane-bound RNA-binding proteins have been identified in the chloroplast of Chlamydomonas. These could serve as the localization factors in mRNA-based protein targeting [202] [186, 188]. At least one of these is involved in translation of the target mRNA and others are activated by light exposure, a condition that also stimulates protein synthesis and targeting [186, 203]. Fourth, most proteins encoded by the chloroplast genomes lack a cleavable N-terminal transit peptide required for protein targeting by the SRP or posttranslational pathways (Section 1). Surprizingly very little is known about how chloroplast proteins get to the translocation complexes in the thylakoid membrane. Finally, in a study in which chloroplast mRNAs and both subunits of the chloroplast ribosome were seen to localize to a specific region of the Chlamydomonas chloroplast for the synthesis of PS II subunits, this localization

also occurred in the presence of lincomycin, a translation inhibitor that clears mRNAs of ribosomes and nascent chains. This result suggest that the localization signals are within the mRNAs and ribosome subunits themselves analogously to template partitioning model for the ER (Section 3) [17, 183].

5.4 Summary

Peroxisomes and chloroplasts have potential to become new frontiers in the exploration of mRNA-based protein targeting and elucidate the long-standing question of how chloroplast genome-encoded proteins are targeted to thylakoid membranes. Dissection of mRNA-based protein targeting in chloroplasts would provide a bacterial-type system in which to study this mode of targeting. Generality of mRNA-based protein targeting is suggested by findings that the translation of mRNAs encoded by the mitochondrial genome in *S. cerevisiae* require translational activators proteins that are bound to the inner membrane and interact with ribosomes [138, 204, 205]. It will certainly be of interest to determine whether or not mRNA-based protein targeting occurs in bacteria, the Archaea, or both kingdoms.

6. Conclusions

mRNA association to the ER surface is an accepted mode for the regulation of intracellular functions. This concept, however, has only recently been transferred to other membranes like the one of mitochondria. In the future it will be of outstanding interest to describe the molecular nature of the association of polysomes or ribosomes to the mitochondrial surface and if there are indeed two distinct binding sites as proposed by Kellems and Butow [108]. Furthermore it will be of importance to describe the molecular composition of the complex formed by Puf3p and if there are other RNA binding proteins besides Puf3p involved in mRNA targeting and localization. At the same time, the analysis of the relation between mRNA targeting, protein translocation and mitochondrial importance in intracellular networks has to be deciphered. For example, the observed localization of mRNAs to the mitochondrial surface leads to the question concerning the demand of cytosolic targeting factors for protein

targeting to the mitochondrial surface. In addition, considering the dynamics of mitochondrial structures it is not yet known whether the mRNA association with the mitochondrial surface is evenly distributed within cells. Similarly, the current understanding of mRNA-based signals involved and their importance is still sparse and almost nothing is known about the dynamics of the mRNA association e.g. with respect to the removal of mRNAs from the membrane surface in response to altered demands of organellar loading. Thus, the understanding of mRNA-localization based regulation of organellar function is just at its beginning and we expect that future studies will lead to the description of yet unknown and may be even unexpected mechanisms.

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Table 1 Glossary of abbreviations

	Term	Explanation
Protein translocation	Cotranslational translocation	The translocation mode, where the translating ribosome is associated with the membrane embedded translocating complex
	Post-translational translocation	The translocation mode, where the protein is translated on cytoplasmic ribosomes and subsequently targeted to the membrane embedded translocating complex.
	Signal sequence, signal peptide, transit peptide	The (mostly) N-terminal positioned amino acid code required for proper targeting to the according membrane and which is recognized by proteinaceous factors. It is cleaved off from the polypeptide after successful translocation. Thereby, signal sequence refers to proteins targeted to the ER, signal peptide refers to the proteins targeted to mitochondria and transit peptide to proteins targeted to chloroplasts. For peroxisomes, signals are assigned as peroxisomal targeting signal 1, 2 or 3.
	Signal recognition particle - SRP	A RNP which recognizes the signal sequence emerging from the ribosomes leading to an SRP induced translation arrest and the targeting of the ribosome to the ER-surface.
	TOM/TIM/TOC/TIC	These are membrane embedded complexes involved in the translocation of the preprotein across the mitochondrial or chloroplast envelope membranes. The abbreviations refer to **t**ranslocon on the **o**uter/inner **m**itochondrial/**c**hloroplast envelope membrane.
RNA distribution	Ribonucleoprotein - RNP	A complex between RNA and proteins, which can form between synthesis of RNAs or subsequently of RNA export from the nucleus and which are involved in modification, packaging and delivery of RNAs, or which form functional complexes like SRP, snoRNP and ribosome.
	cis element / ZIP-code	A sequence element on the mRNA (mostly in the UTR's) that acts on the same molecule (cis) either in localization or recruitment of RNA-binding proteins
	MLR value	Value of mitochondrial localization of mRNAs as determined first by Marc *et al.* [14] in their transcriptome wide screen. A high MLR value means strong co-localization of a mRNA with the mitochondria.

Figures

Figure 1. The different modes of mRNA associations to the ER-membrane surface.

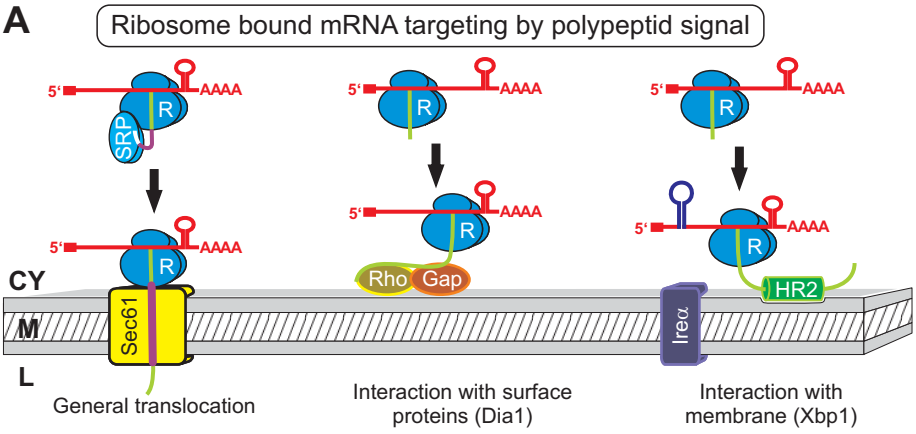
a) mRNA association mediated by the emerging polypeptide. The association of the ribosome with the ER is generally mediated by SRP connection to the Sec61 complex (left). SRP recognizes the signal sequence emerging from the ribosomal exit tunnel, stalls translation and targets the ribosome-mRNA-nascent chain complex to the ER surface via docking to the SRP receptor Sec61 which sits in the membrane. Albeit less frequent, interaction of the ribosome with the ER membrane can be achieved as a byproduct of nascent chain interaction with ER proteins (middle). Mammalian Dia1 is a cytosolic protein that interacts with a Rho-GTPase that sits on the ER membrane thus mediating the connection of ribosome to the ER. Furthermore, the ribosome-ER interaction is used for regulation of the UPR (right). At least in mammalian cells, the mRNA coding for Xbp1p is already found at the ER membrane even the UPR is not switched on. The non-spliced intron leads to translation of the hydrophobic HR2-segment that interacts with the ER membrane. Thus, both the translational arrest and the wrong polypeptide lead to targeting of the ribosome to the ER.
b) Ribosome association enforced by mRNA elements for import. Additional to polypeptide based ribosome targeting to the ER, the mRNA itself can mediate a connection to the ER membrane. Examplified here is the mRNA coding for Pmp1 (left), which contains a mRNA motif in the 3' UTR that folds to a hairpin structure that mediates ER-interaction through a today not known protein factor. Since Pmp1p engages the secretory pathway, the protein is translated into the ER through Sec61 after docking of its mRNA to the ER surface. For the UPR in yeast, the stalled ribosome-mRNA-nascent chain complex is only associated with the ER membrane during UPR. Interestingly, this association is mediated by a mRNA element in the 3' UTR that connects to Ireα (right).
c) Ribosome independent association of mRNA for regulation (3.2 – Bip1p). mRNAs coding for cytosolic proteins are likely distributed close to the perinuclear surface serving to deliver the messengers to the translation machinery sitting at the ER membrane. This is exemplified by the *ASH1* mRNA which is delivered to the bud tip ER in yeast by She2p (ER connection) and Puf6 (translational repression), both binding to distinct motifs in the 3' UTR of *ASH1* mRNA.

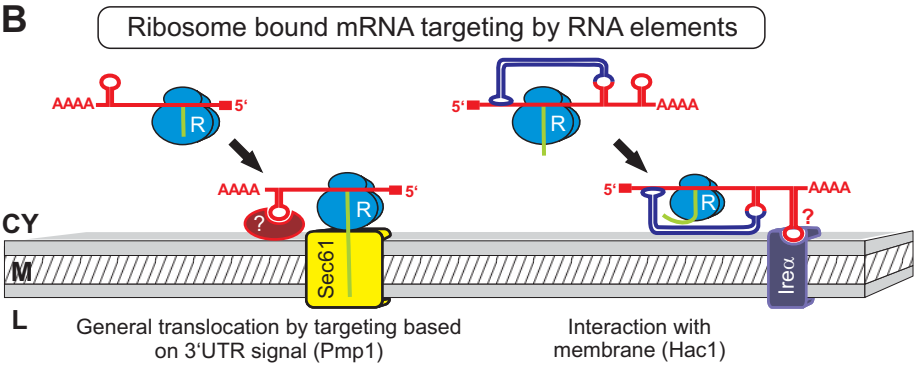
Figure 2. The different modes of mRNA associations with the mitochondrial surface.

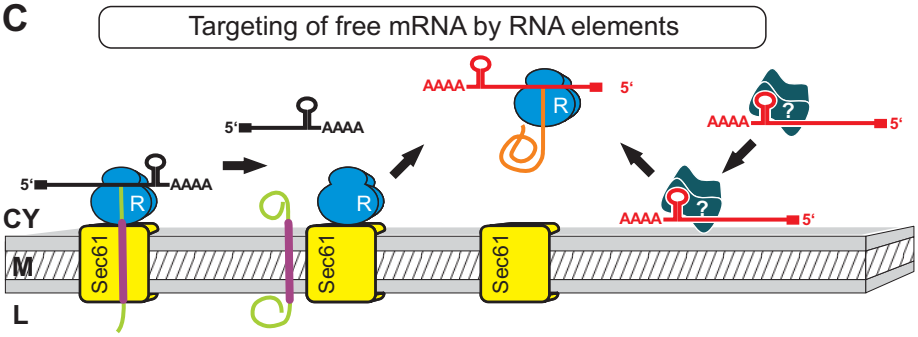
a) mRNA association through the emerging polypeptide. The majority of mitochondrial proteins is translated on cytoplasmic ribosomes and imported post-translational via the connection to molecular chaperones (Hsp70, Hsp90) that keep the pre-protein in an unfolded state (upper panel). The precursor is recognized by the TOM-complex, imported into mitochondria and gets further processed by the mitochondrial processing peptidase (MPP). However, for some proteins it is indispensable that they are imported cotranslational (lower panel), since the fully translated pre-protein would for unimportable aggregates in the cytoplasm (here exemplified for Fumarase). Thus, the ribosome is targeted to the outer membrane of mitochondria through the transit peptide.

b) Direct association of mRNAs with the mitochondrial surface. mRNAs can associate directly with the outer membrane of mitochondria. During the transcriptome studies two classes of mitochondria targeted mRNAs were developed. Class I mRNAs (Puf3p-dependent) have a distinct Zipcode in their 3' UTR that is recognized by Puf3p that subsequently targets these mRNAs to the outer mitochondrial membrane by connecting to Mmd12p (left). Class II mRNAs (Puf3-independent) are targeted to the outer mitochondrial membrane by a yet unidentified factor (right).

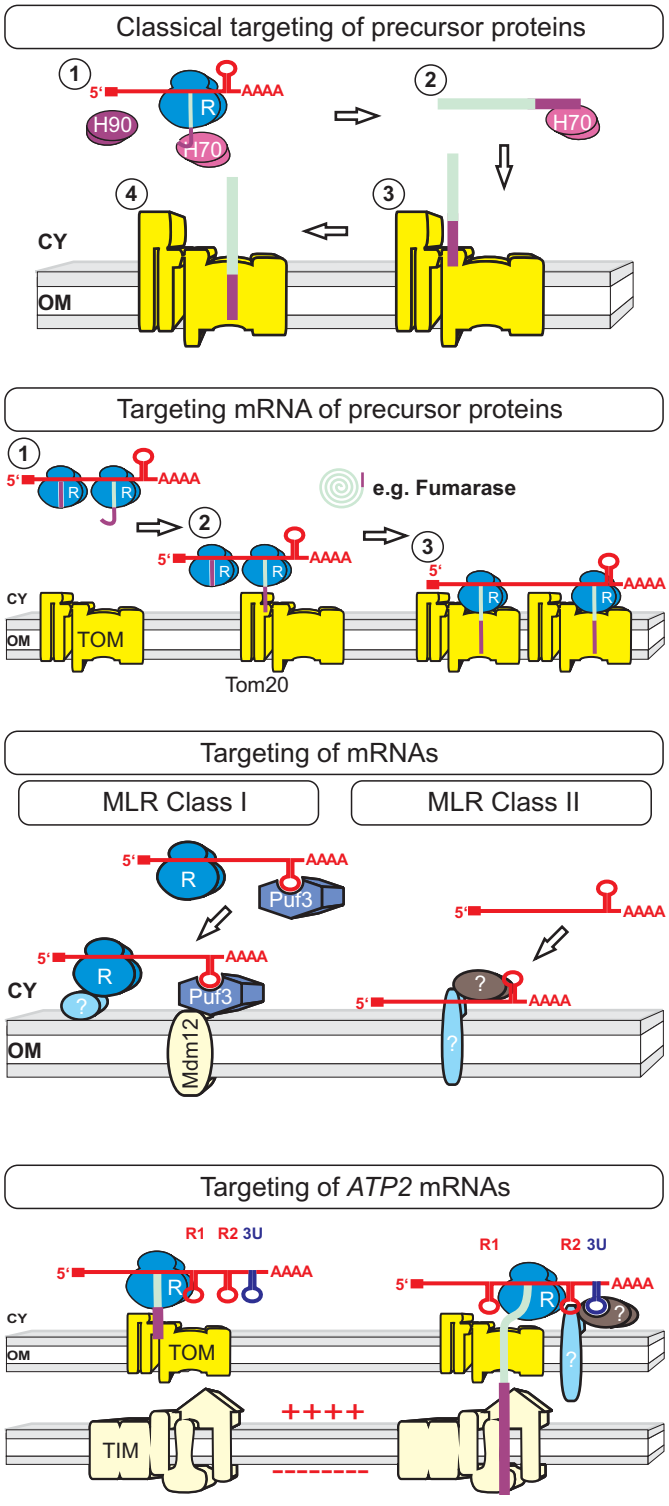
c) Targeting of the *ATP2* mRNA to the mitochondrial surface. One of the deeper studied examples for an mRNA that associates with the mitochondrial membrane is the *ATP2* mRNA that belongs to the class II mRNAs. It was shown that the complete mitochondrial association of this mRNA is achieved by a cooperative mechanism involving the elements in the 3' UTR (3 U), in the mitochondrial targeting sequence (R1) and the ORF (R2). The emerging polypeptide of Atp2p and the inner membrane potential alone are necessary but not sufficient to target the mRNA-ribosome complex to the vicinity.

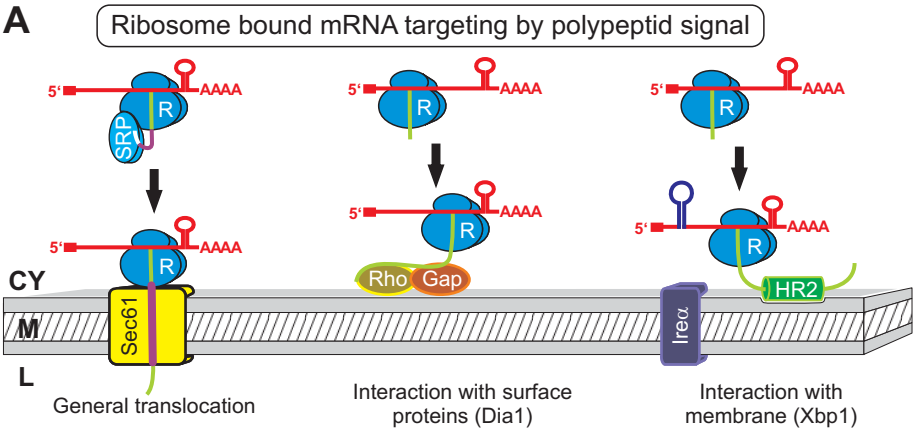


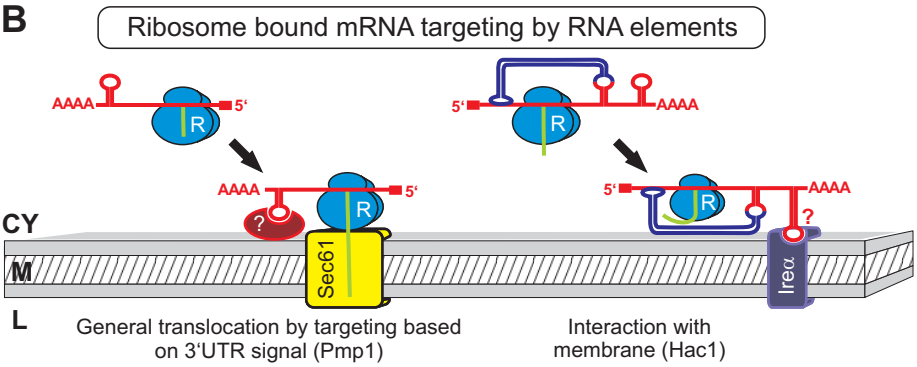


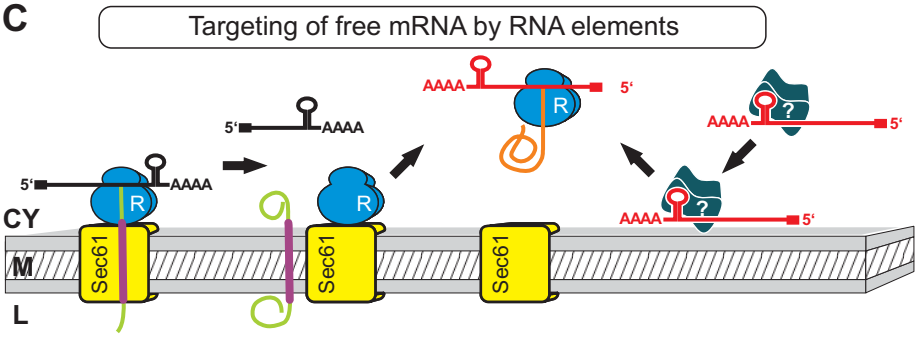


Process for mRNA storage or transport based on ER-membrane movement (Ash1/Bip)


Classical targeting of precursor proteins
1
2
3
4
5'
AAAA
R
H90
H70
CY
OM
Targeting mRNA of precursor proteins
e.g. Fumarase
TOM
Tom20
Targeting of mRNAs
MLR Class I
MLR Class II
Puf3
Mdm12
?
Targeting of ATP2 mRNAs
R1
R2 3U
TOM
TIM
++++







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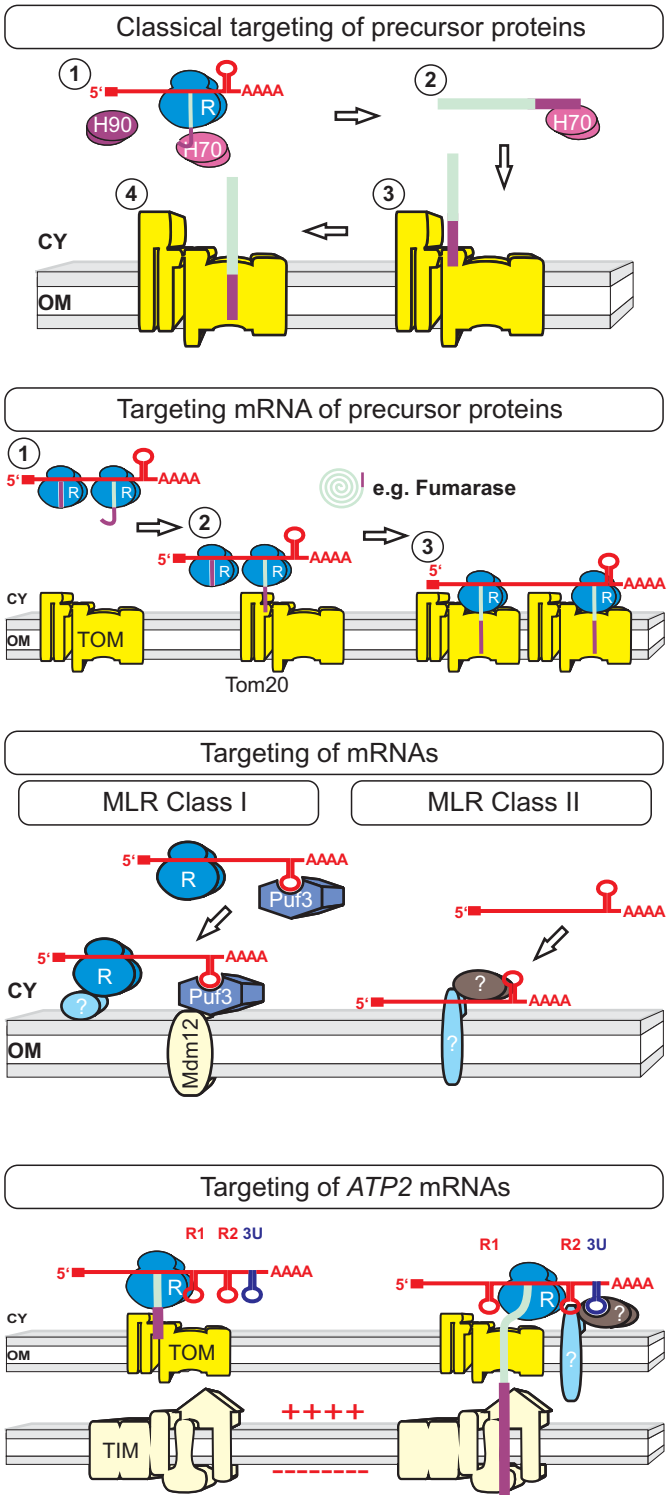
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++++
