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## Review

## Posttranscriptional control of mitochondrial biogenesis: Spatio-temporal regulation of the protein import process

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## ABSTRACT

**This review focuses on the posttranscriptional processes which govern mitochondrial biogenesis, with a special emphasis on the asymmetric localization–translation of nuclear-encoded mRNAs as an important regulatory step of the protein import process. We review how spatio-temporal mRNA regulons help to elicit timely, versatile, and coordinated intracellular processes to assemble mitochondrial structures. Our current knowledge on the mitochondrial import of respiratory chain assembly factors and the role of the ribonucleic acid (RNA) binding protein Puf3 are presented. A connection with the target of rapamycin signalling pathway may explain how respiratory chain assembly senses environmental conditions via the protein import machinery.**

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### 1. Introduction

Most of the mitochondrion proteome is made of nuclearly-encoded preproteins, translated in the cytosol, which have to be imported, processed, assembled into large macromolecular complexes and located to the proper mitochondrial compartment. The abundance, morphology and functional properties of mitochondria are finely tuned to meet cell-specific energetic and metabolic demands. It is generally assumed that, in different organisms, this tuning is largely achieved in the nucleus, at the level of transcriptional regulation [1–3], whereas the mitochondrial import machinery is simply in charge of the protein sorting inside mitochondrial compartments [4,5]. However, *in vivo* studies in budding yeast and HeLa cells have stressed the importance of the processes preceding the import step [6–8]. It seems clear, long after the pioneering work of Butow et al. [9], that some nuclearly-encoded proteins can be co-translationally imported into mitochondria. Global transcriptome studies have demonstrated that

roughly half of the mRNAs coding for mitochondrially-localized proteins are asymmetrically localized to the vicinity of mitochondria [10,11]. This implies that gene-specific post-transcriptional processes cooperate with the mitochondrial import machinery. The recent demonstration of a role of Tom20, a subunit of the outer membrane translocase, in the co-translational import process [12,13], raises the possibility that the protein import can be modulated via precise mRNA localization and site-specific translation.

This review will focus on the role of mRNA localization and site-specific translation in the control of mitochondrial protein import, as an important posttranscriptional control of gene expression. The specific case of the spatio-temporal control of the assembly of respiratory chain complexes during the yeast metabolic cycle will be emphasized, as an emblematic example of cooperation between transcriptional and co-translational events. The mechanisms, that possibly link the co-translational protein import at mitochondria and cytosolic signals connected to cell growth and energetic demand, will be discussed.

### 2. Key examples of mitochondria import regulations

Studies carried out in different eukaryotic systems have pointed out the mitochondrial protein import process as a potentially important level of regulation of gene expression and mitochondrial activity. An exciting example of regulated protein import into yeast

*Abbreviations:* RNA, ribonucleic acid; ADP, adenosine diphosphate; ATP, adenosine triphosphate; rRNA, ribosomal RNA; TOR, target of rapamycin; OXPHOS, oxidative phosphorylation; MLR, mitochondrially localized RNA; MTS, mitochondrial targeting sequence

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mitochondria is the case of fumarase [14,15]. Fumarase is asymmetrically distributed between the cytosol and mitochondria, depending on the metabolic state of the cells. When the glyoxylate shunt is shut off, and therefore fumarase is no longer required in the cytosol, the protein accumulates exclusively in mitochondria. The mechanisms controlling this differential distribution are not known, though the authors favour a change in fumarase folding, which would modulate the rate of translocation into mitochondria.

In plant cells the mitochondrial import of proteins is a dynamically regulated process that is influenced by the light–dark cycle, wounding, and developmental cues [16]. In cardiac muscle cells, thyroid hormone (T3) treatment dramatically increases the rate of mitochondrial protein import [17]. This rate can also be altered in mitochondria from senescent animals [18] and mutations that disrupt the proper functioning of the import machinery can lead to oxidative stress, neurodegenerative diseases and metabolic disorders (reviewed in [6]). Additionally, external factors, like Rubella virus, can inhibit mitochondrial import via the action of a capsid RNA-binding protein which probably targets important component of the translocation apparatus [19].

### 3. Connections between protein import and mitochondrial metabolic activity

Recent studies have pointed out relationships between the import machinery and the establishment of mitochondrial functions. This was especially well addressed in plants. Rice shows substantial tolerance to anaerobic conditions and is able to germinate and grow for days in the absence of oxygen. Studies in relatively mature rice tissues have shown that, in aerobic conditions, an increase in the import capacity is correlated with an increase in the abundance of the cytochrome bc1 complex, thus revealing a link between the activity of respiratory chain complexes and the protein import apparatus [20,21]. Interestingly, in plants, the mitochondrial processing peptidase is part of the cytochrome bc1 and is repressed both at transcript and protein levels, under anaerobic conditions, providing a direct connection between mitochondrial protein import and processing, and the respiration rate. Physical interactions, mediated by the Tim21 protein, were observed in the budding yeast between the inner membrane translocase TIM23 and subunits of the respiratory chain complexes [22,23]. Also the adenosine diphosphate/adenosine triphosphate (ATP) carrier Aac2, which plays a central role in the mitochondrial metabolism, exists in physical association with the cytochrome *c* reductase (cytochrome bc1)–cytochrome *c* oxidase (COX) super-complex and its associated TIM23 machinery [24].

### 4. Mechanisms that control the mitochondrial protein import machinery

The outer membrane protein translocase, that functions as a general entry gate for preproteins into mitochondria is, *a priori*, a good target for import regulation processes. Up-regulation of the protein import process may result from an increased synthesis of the diverse translocases. For instance, in plants, the level of Tom20 was found to positively correlate with import capacity [25]. More recent studies [20] have shown that transcript levels of mitochondrial protein import components are unaffected by oxygen availability, indicating that the abundance of these proteins, which is higher in anaerobic conditions, is regulated at a post-transcriptional level. In budding yeast, the expression of genes associated with protein import is positively correlated with the growth rate together with a substantial fraction of the genes associated with translation, ribosome biogenesis and ribosomal RNA (rRNA) metabolism [26]. This perception by the mitochondria

of the nutritional environment of the cell should depend on target of rapamycin (TOR) signalling. TOR is a conserved signalling pathway which controls cell growth by integrating multiple environmental inputs, including nutrients availability, growth factors and stress. TOR exists in two different protein complexes: TORC1 and TORC2. Both complexes exhibit protein kinase activities and regulate many key cellular processes, including translation, ribosome biogenesis and basal metabolism. However the relationships between TOR and the mitochondria are complex. Although inhibition of the TOR pathway leads to a global decrease of the cytoplasmic translation [27], *tor1* null cells exhibit a higher rate of mitochondrial translation and an increase in the expression of several mitochondrially encoded oxidative phosphorylation (OXPHOS) components [28]. Accordingly, the treatment of budding yeast with rapamycin, an inhibitor of TORC1 activity, increases the expression of genes involved in the TCA cycle, mitochondrial ribosome biogenesis, and assembly of the OXPHOS complexes [29]. In mammalian cells, hypoxia represses mTOR (mammalian TOR) as if the cells were exposed to rapamycin [30] and mTOR activity is regulated in a redox-sensitive manner [31], which establishes a homeostatic regulation loop between mTOR and mitochondria [32]. This connection between TOR activity and membrane-linked translation may be an important aspect of the mitochondrial import regulation, since many mRNA encoding mitochondrial proteins are translated by ribosomes present at the external membrane of mitochondria (see below). The recent demonstration, in a leukemic cell line, that mTOR directly controls mitochondria function, while being localized to the outer membrane of the mitochondria [33], strongly supports a direct action of TOR on proteins located at the mitochondrial outer surface. In yeast, this effect could be mediated by the Sch9p kinase, which was shown to be the main target of TORC1 [34], and the inactivation of which mimics the effects of an inhibition of TOR activity on respiration and life span [28,35]. Altogether these observations suggest that TOR signalling could be a key actor in the regulation of the mitochondrial import process.

### 5. Mitochondrial co-translational import distinguishes groups of genes

The site-specific translation of localized mRNA is a fast and efficient way of reacting to external stimuli, with the added benefit of providing spatial resolution to the cellular response [36]. Several genome-wide studies [10,11,13] have shown that about half of the mRNAs encoding mitochondrial proteins are enriched in the vicinity of mitochondria, presumably to facilitate protein transport. Interestingly, these mitochondrially localized RNAs (MLRs) can be mechanistically split in two classes: class I mRNAs (MLR I), whose asymmetric localization depends on the presence of the RNA-binding protein Puf3, and class II mRNAs (MLR II), which are localized to the vicinity of mitochondria independently of the presence of the *PUF3* gene [10]. Finally, a third class (class III) groups the mRNAs encoding mitochondrial proteins, which are not translated at the vicinity of mitochondria.

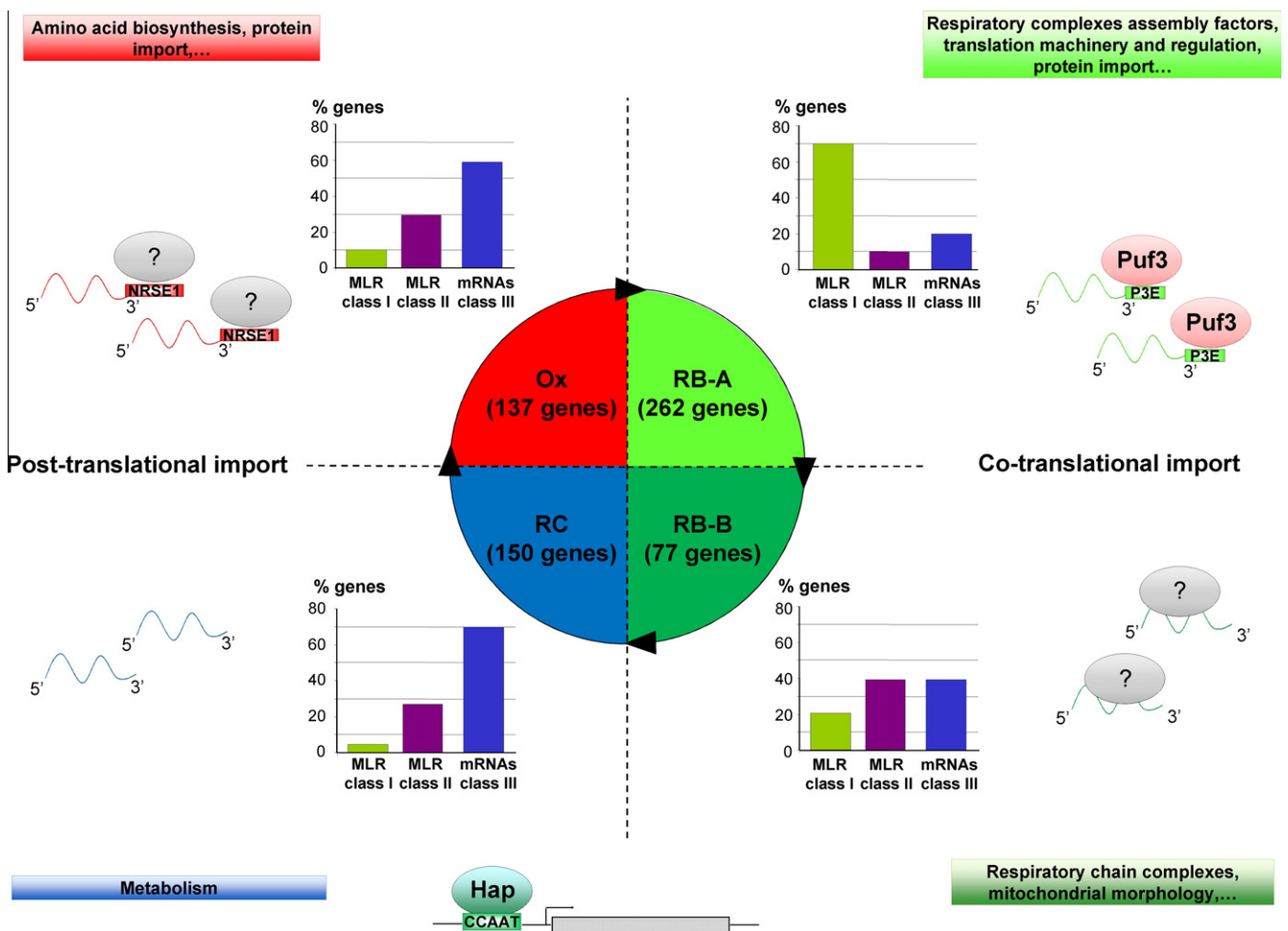
### 6. Parameters that control the mRNA asymmetric localization to the mitochondria

At least four elements are known to control the peri-mitochondrial mRNA asymmetric localization, which are more or less important, depending on the MLR class that is considered: (i) the mitochondrial targeting sequence (MTS), which is located at the N-terminus of the nascent pre-proteins, is more than a general protein feature that interacts with the Tom20 translocase to favour mitochondrial addressing. It also acts to regulate this translocation

process. Thus, the MTS can either tilt the balance between mitochondrial and cytosolic dual localization [15], or guide the mRNA asymmetric localization to the vicinity of mitochondria [12,13]. However, the MTS is present in many mitochondrial proteins independently of the three classes described above [37], and therefore is not sufficient to explain the site-specific translation of MLRs, (ii) the outer membrane protein, Tom20, was shown to mediate localization of mRNAs to mitochondria in a translation-dependent manner [13]. Absence of this major import receptor alters the repertoire of mRNAs localized to the vicinity of mitochondria, (iii) the translational machinery was observed to be required to maintain MLRs next to mitochondria [12,13]. In mammals it was clearly demonstrated that ribosomes specifically bind to mitochondria via protease-sensitive proteins on the outer membrane [38] and it is reasonable to hypothesize that ribosomes are involved in the direct interaction of some mRNAs with mitochondria [9],

(iv) Puf3 and probably other unidentified mRNA binding proteins are required to maintain mRNA asymmetric localization to the vicinity of mitochondria. The MLR I class largely overlaps the Puf3 target mRNAs previously identified [39] and the importance of the Puf3 binding site in the 3'UTR of some MLR I members for their mitochondrial asymmetric localization was experimentally demonstrated [10].

Recent studies have shown that localization of some MLR I to mitochondria depends on both Puf3p and Tom20p [13]. Accordingly, a *tom20Δ puf3Δ* strain exhibited growth defects under conditions where fully functional mitochondria are required [13]. While it was strongly suggested that Tom20p and Puf3p are both localized to the mitochondrial outer membrane [40], they do not seem to interact with each other, at least in standard growth conditions [13]. However, Puf3 controls the Tom20 mRNA localization next to mitochondria [10] and the deletion of *TOM20* considerably



**Fig. 1.** The mitochondria cycle. When grown under continuous, nutrient-limited conditions, budding yeast exhibits robust, highly periodic cycles (from 40 [59] to 300 min [43]). Clustering analyses [43,44] of gene expression of 656 genes encoding mitochondrial proteins have emphasized four different phases during the metabolic cycle: Ox (oxidative, 137 genes mostly involved in amino-acid synthesis), RB-A (reductive building A, 262 genes coding for assembly and translation factors), RB-B (reductive building B, 77 genes coding mainly for respiratory chain subunits) RC (reductive charging, 150 genes, coding for TCA cycle enzymes and other metabolic pathways). The percentages of genes in each of the four phases, which belong to the MLR class I (green bars), MLR class II (purple bars) and mRNA class III (blue bars) categories, are represented by histograms. The cis- and trans- regulatory elements which have been demonstrated or predicted to play a role in the yeast mitochondrial cycle [44] and in MLR site-specific translation [10] are represented as follows. Puf3 (upper right) recognizes the P3E motif in the 3'UTR of its targets RNA and plays a role in targeting them to the mitochondrial surface [10]. These Puf3 targets are MLR class I which are mostly expressed during the RB-A phase. The Hap complex and the Hap4 transcription factor (bottom middle) recognize the CCAAT box in the promoter of its target genes. These genes are mostly expressed during the RB-B or the RC phases. The MLR class II mRNAs (bottom right) are spread between the RB-B, the RC and the Ox phases but represent the major class in the RB-B phase. In the particular case of *ATP2* mRNA, it has been shown that the mitochondrial asymmetric localization depends on cis-regulatory sequences located into the open reading frame [12]. The proteins recognizing these sequences are unknown to date. The class III mRNAs (on the left) are spread between the RB-B, the RC and the Ox phases, and they represent the large majority of the genes expressed during the RC and the Ox phases. The class III mRNAs expressed during the Ox phase (upper left) share a special feature: most of them have a NRSE1 motif in their 3'UTR. NRSE1 has been characterised by in silico analyses [54]. The role of this sequence and its corresponding RNA binding proteins are unknown. Finally, the main mitochondrial functional categories enriched in each phase of the cycle are indicated at each corner of the figure (based on [44]).

increases the level of *PUF3* mRNA and protein [13]. This increase could be an adaptation to the mislocalisation of Puf3 in the cytosol, that is observed in *tom20Δ* strains [13].

A simple scenario can describe the main steps controlling the co-translational processing of MLRs: (1) interaction of Tom20p with the emerging pre-protein through its MTS, (2) translation of the downstream ORF, (3) interactions of additional RNA-binding factors which, by interacting with ORF-contained motifs [12] or signals in the 3'UTR (like Puf3-motif) [10], can favour mRNA-mitochondria interactions, while participating in the regulation of mRNA translation and stability. An essential aspect of this process is the tight control of translation which has to be blocked until mRNA has reached its appropriate localization and whose rate has to be severely modulated to maintain ribosomes in close proximity with mitochondria. In the MLR II member *ATP2*, the translation of two elements in the coding sequence has been shown to be essential for mRNA anchoring to the mitochondrial surface [12]. This translation regulation of MLRs may also be achieved by RNA-binding proteins, such as Puf3 in the case of the MLR I members, which interacts both with the external side of mitochondria [40] and with P-bodies [41], and therefore could control the localization and the translation/degradation balance of its target mRNA. Alternatively, such RNA binding proteins could coat the mRNAs in the nucleus during mRNP formation or straight after cytoplasmic export to ensure translational repression during transport, as proposed by Lithgow et al. [42]. In conclusion, site-specific mRNA translation seems important to precisely control both the localization and the rate of cotranslational protein import into mitochondria.

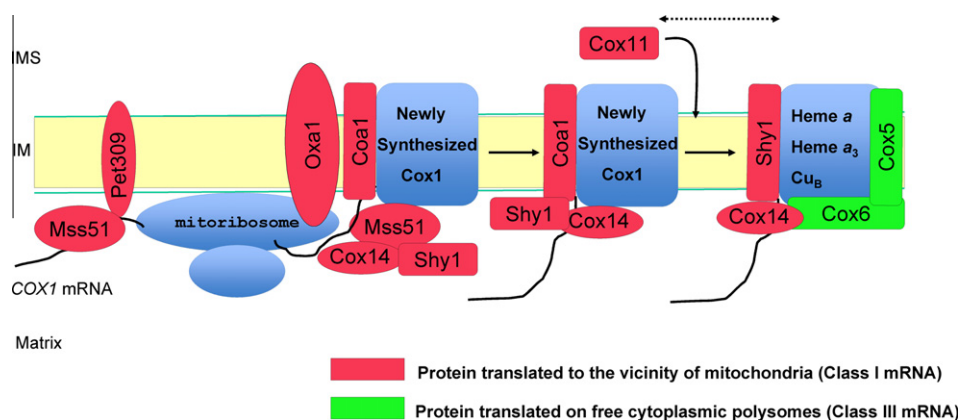
## 7. Protein import and the spatio-temporal regulation of mitochondria biogenesis: a complex interplay between transcriptional and post-transcriptional events

The cotranslational import of groups of mitochondrially-localized proteins challenges the previous dogma that placed transcriptional control at the centre of gene regulation and relegated translation to a more passive role. Co-regulated groups of genes were precisely identified in Yeast Metabolic Cycle data obtained by transcriptome analysis of metabolically synchronized yeast cell cultures [43]. The time-dependent expression of groups of nuclear genes encoding mitochondrial proteins strikingly correlates with their mode of mitochondrial import [44]. The most salient feature is probably the identification of a group of genes (named RB-A in

Fig. 1), whose mRNA amount increases at a very early step of the Yeast Metabolic Cycle. These 262 mRNAs are mainly (70%) translated to the vicinity of mitochondria and contain a canonical Puf3 binding site in their 3'UTR [39,44]. They correspond to functions required for the construction of mitochondria (assembly factors, ribosomal proteins, translation and transcription regulators). Interestingly this group of genes, whose site-specific translation seems to be tightly regulated, do not seem to be transcriptionally regulated [45] and no evident consensus motif could be found in their promoter region [44], suggesting that their up-regulation is mainly due to a reduced mRNA degradation. The situation is more classical for the following group of genes (RB-B in Fig. 1), which code mainly for respiratory chain subunits, exhibit a transcriptional regulation by Hap transcription factors [46] and a controlled mRNA asymmetric localization [10]. The best documented case of transcriptional regulation assisted by a posttranscriptional regulation process is that of *ATP2*, which belongs to the RB-B group, has a bona fide Hap binding site in its promoter and whose translation, to the vicinity of mitochondria, is tightly regulated [12].

## 8. Respiratory chain assembly factors: an interesting Puf3 post-transcriptional regulon

The protein import process, through the co-translational import process, is able to funnel functionally connected groups of proteins according to a spatio-temporal program. Several groups of proteins are concerned but the case of the respiratory chain assembly factors is especially convincing. The biogenesis of the respiratory chain complexes is critical for cellular bioenergetics and as such it is tightly regulated to cope with cellular and environmental conditions. Biogenesis of cytochrome c oxidase (COX), the last enzyme of the mitochondrial respiratory chain represents a case study by the importance and complexity of this multimeric enzyme [47,48]. In the budding yeast, COX is formed by 13 different subunits of dual genetic origin (10 nuclear and 3 mitochondrial). Its assembly involves at least 27 assembly factors which control different assembly steps. For instance, the mitochondrion-encoded Cox1 subunit is incorporated within one early assembly intermediate containing the assembly factor Mss51 [49]. Subsequently, Cox1 maturation involves the progression to downstream assembly intermediates requiring the assembly factors Coa1, Cox14 and Shy1 [50], and COX biogenesis continues under the control of a sophisticated ballet of assembly factors. Each respiratory chain complex has its own set of assembly factors, only Oxa1, the first



**Fig. 2.** Early steps of COX assembly: assembly factors are co translationally imported. This model is adapted from Khalimonchuk et al. [49] and Mick et al. [60]. When the Cox1 subunit is translated inside mitochondria, the first protein complexes are formed with the nuclear-encoded assembly factors Mss51, Pet309, Oxa1, Coa1, Cox11, Shy1. All these factors are likely to be co translationally imported since their mRNA is closely associated with mitochondria and contains a Puf3 binding site in their 3'UTR (MLR I). Constitutive Cox5 and Cox6 subunits are probably imported posttranslationally (Class III mRNA). Note that among the 25 identified assembly factors involved in COX assembly, all but one (Cox16) belong to the MLR I class.

discovered assembly factors [51], is shared by the three different respiratory complexes (III, IV and V). All these assembly factors share similar post-transcriptional properties which are tightly connected with their mitochondrial import process and which are illustrated in Fig. 2: (1) their mRNA is asymmetrically localized, 31 of the 32 mRNAs coding for respiratory complexes assembly factors are localized to the vicinity of mitochondria, (2) most of them (29/32) have a Puf3 binding site in their 3'UTR sequence which has been shown to be functionally active in many cases [10,39], (3) 25/32 of these mRNAs are more abundant in the very early beginning of the yeast metabolic cycle strongly suggesting that, in metabolically synchronized cells, assembly factors are produced before respiratory chain subunits and thus constitute the corner stones of mitochondria biogenesis.

To summarize, whereas the regulation of assembly factor coding genes appeared quite heterogeneous and difficult to rationalize [45], the analysis of transcriptome data from metabolically synchronized strains [43], together with data concerning the topological properties of their mRNAs [10,44], have revealed that assembly factor mRNAs share common properties: they are regulated mostly post-transcriptionally both by their translation mode (localization, controlled rate) and by their stability. In that respect they constitute one of the best documented case of a decay regulon [52] or, more generally, a post-transcriptional regulon which coordinates the localization, translation and stability of mRNAs [53].

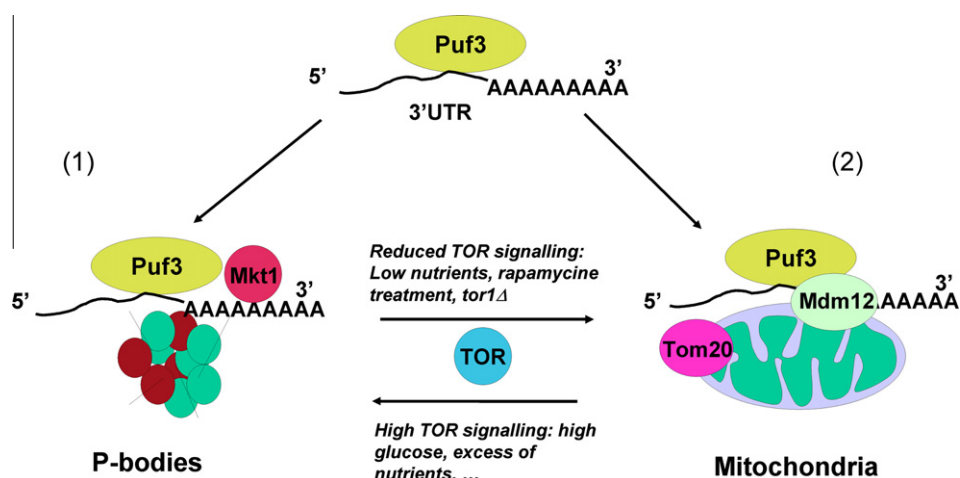
### 9. Could TOR signalling pathway regulates mitochondrial import via Puf3?

The above scenario may offer some clues for better exploring the regulatory mechanisms which control mitochondrial biogenesis via the mitochondrial import process (Fig. 3). It was recently proposed that the role of Puf3 in mRNA translation and stability could be connected to the Mkt1 protein, which interacts with the Poly(A)-binding protein associated factor Pbp1 [41]. Moreover, rapamycin treatment has been shown to reduce the ability of

Puf3 to control mRNA stability [54]. Finally, in a leukemic cell line, inhibition of mTOR has immediate effects, which cannot be explained by transcriptional changes, on carbon and mitochondrial metabolism by acting directly at the mitochondrial outer membrane. It is tempting to propose that, in the yeast, Puf3, which is also located at the mitochondrial outer membrane [40], might be a direct phosphorylation substrate for TOR signalling. In this model, two distinct states of Puf3-mRNA complexes would exist: (i) a repressive complex associated with P-bodies, and (ii) an activating complex at the mitochondria, engaged in co-translational import. The balance between these states could be achieved by TOR, which is a negative regulator of Puf3 function and mitochondrial biogenesis (Fig. 3). This would allow mitochondria to swiftly adapt to the energetic, metabolic and signalling demand of the cell, without relying on time-consuming transcriptional processes. It is important to mention that the functional homologue of Puf3 in human cells is unknown to date. None of the human pumilio proteins PUM1 and PUM2 interact specifically with mRNAs encoding mitochondrial proteins [55–57]. However, further experimental work is needed to exclude a role of PUM1 and PUM2 in mRNA localisation to the mitochondria.

### 10. Concluding remarks

Importance of the mitochondrion in the cell physiology implies that this organelle should have the capacity to swiftly adapt its properties to the cellular or extracellular context. The transcriptional regulation of nuclear genes coding for mitochondrially localized proteins is certainly important, but it cannot account for a fast adaptive response. Recent studies have underscored the importance of a co-translational import process concerning a large set of proteins important for mitochondrial biogenesis. This process, which relies on a tight translational regulation of localized mRNAs, can rapidly respond to cellular conditions while giving the possibility to orchestrate complex assembly at discrete foci on the mitochondrial membrane. TOR signalling might play a central role in



**Fig. 3.** Metabolism and co-translational import regulation. (1) Puf3 may serve as a regulatory signal in processes taking place in P-bodies [41]. Puf3-mRNA complexes can be stored in P-bodies where they are translationally silenced and either degraded or released back in the translating pool [61]. (2) Reduced nutrient sensing TOR pathway or dietary restriction increase translation of nuclear-encoded mitochondrial genes [62,63]. Rapamycin alters P-bodies-linked Puf3 properties [54] and may favour the formation of co-translationally active mRNA-Puf3-Tom20 complexes. Class I mRNAs are translated to the vicinity of mitochondria under the control of the mRNA-binding protein Puf3 [10]. Puf3 and the outer membrane translocase Tom20 are both necessary for an asymmetric mRNA localization [12,13]. Puf3 interaction with the mitochondrial surface is dependent on Mdm12 [40]. This model is speculative and many of the factors involved in this equilibrium have to be studied. For instance, Mkt1 is a protein which interacts with the Poly(A)-binding protein associated factor, Pbp1, during translation and can either regulate translation [64] or control the stability of Puf3-dependent transcripts by regulating P-body abundance [41]. Mkt1 is a good candidate to play an important role in the P-bodies-Mitochondria equilibrium of Puf3 [41]. In addition, the fact that *MKT1* allele reduces the formation of *petite* colonies and compromised the growth of *petite* cells [65] is a strong indication of its role in mitochondria biogenesis. Though several other putative functions for Mkt1 have been mentioned mostly in connections with environmental conditions [66,67], its role in mitochondria biogenesis seems predominant. Yeast TOR is also a very important factor in the control of Puf3 properties [54] and the recent finding that, in a leukemic cell line, mTOR can be localized in a complex comprising the outer mitochondrial membrane proteins Bcl-xl and VDAC1 is certainly worth noting [33]. Moreover, in mammals mTOR also controls mitochondria biogenesis through the YY1-PGC-1 $\alpha$  transcriptional complexes [68] thus connecting transcriptional and post transcriptional regulation.

this regulation via modification of mRNA-binding proteins like Puf3 [54]. Recent data showed that reduced TOR signalling extends yeast chronological life span by increasing oxygen consumption. This is, in part, due to a up-regulation of the translation of mtDNA-encoded OXPHOS subunits [58]. TOR could then be an important element to connect mitochondrial protein import and the synthesis of mitochondrial proteins. Interestingly Puf3 controls the translation of many important mRNAs including *TOM20* mRNA, which codes for an important component of the outer membrane translocator. The Tom20-Puf3 couple, which may sense the environmental context, seems to be important to regulate the in vivo import of many mitochondrial proteins [13]. Unquestionably the Tom20-Puf3 regulon is not the only protein association in charge of the mitochondrial import regulation but it is a paradigmatic example which certainly opens the way to new approaches to study mitochondrial biogenesis and which may be relevant to better understand dramatic pathological situations.

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