Chapter 5
Mechanisms and Control of Protein Synthesis in Yeast Mitochondria

Steffi Gruschke and Martin Ott

Abstract In the yeast *Saccharomyces cerevisiae,* eight proteins are encoded by the mitochondrial genome. Seven of them are core catalytic subunits of complexes III and IV of the respiratory chain and the ATP synthase and thus essential for oxidative phosphorylation (OXPHOS), while one protein is soluble and a constituent of the small subunit of mitochondrial ribosomes. The expression of these proteins is mainly controlled posttranscriptionally by so-called translational activators. These nuclear-encoded factors act on the 5′-untranslated region (UTR) of their specific client mRNA and stimulate translation. In addition, translational activators play multiple roles in regulation and organization of mitochondrial protein synthesis. The mitochondrial OXPHOS complexes are assembled from subunits encoded by both the nuclear and the mitochondrial DNA. During the biogenesis of OXPHOS complexes, translational activators help to coordinate cytosolic and mitochondrial translation by adjusting mitochondrial protein synthesis to levels that can successfully be assembled. This chapter summarizes the current knowledge about how mitochondrial protein synthesis in the model organism *Saccharomyces cerevisiae* is coordinated with OXPHOS complex assembly.

5.1 Mechanisms of Protein Synthesis in Yeast Mitochondria

5.1.1 The Mitochondrial Genome in Yeast

Mitochondria are key organelles of eukaryotic cells that participate in important metabolic processes like the TCA cycle, fatty acid oxidation, and amino acid

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degradation; they also play important roles in a variety of biosynthetic pathways and contribute to regulation of cellular signaling and apoptosis. Mitochondria evolved 2 billion years ago when an archaeal cell established a symbiotic relationship with aerobic bacteria (Sagan 1967; Gray 1989). In the course of evolution, most of the former bacterial genes were transferred to the nuclear DNA. Concomitantly, the organellar genetic code developed away from the standard genetic code, so that the codon usages differ significantly between both systems. The most obvious alteration is that the universal stop codon TGA is translated into tryptophan (Barrell et al. 1979; Fox 1979). Today, mitochondrial genomes of fungi and higher eukaryotes typically code only for a small number of genes. These include genes for proteins of OXPHOS complexes as well as tRNAs and rRNAs of the mitochondrial translation machinery. In the yeast *Saccharomyces cerevisiae,* eight proteins are encoded in the mitochondrial DNA (mtDNA), 24 tRNAs as well as the rRNA of the small (15S) and large (21S) subunit of the mitochondrial ribosome (Borst and Grivell 1978). Of the eight mitochondrially encoded proteins, seven represents very hydrophobic core subunits of OXPHOS complexes (cytochrome *b* of the bc_1 complex, Cox1, Cox2, and Cox3 of cytochrome oxidase and Atp6, Atp8, and Atp9 of the ATP synthase) located in the inner membrane of mitochondria, while one is a soluble protein and a component of the small mitochondrial ribosomal subunit (Fig. 5.1). These proteins are synthesized on mitochondrial ribosomes which are, unlike bacterial ribosomes, permanently associated to the inner membrane (Fiori et al. 2003; Ott et al. 2006; Prestele et al. 2009), thereby allowing co-translational membrane insertion of the hydrophobic OXPHOS subunits (Hell et al. 2001; Jia et al. 2003; Szyrach et al. 2003; Ott and Herrmann 2010).

5.1.2 The Mitochondrial Ribosome in Yeast and the Process of Translation

The central components of the translation machinery in mitochondria are mitochondrial ribosomes. Due to the endosymbiotic origin of the organelles, it has long been assumed that mitochondrial ribosomes closely resemble the bacterial particles. However, although this is true for certain aspects like catalytic properties or sensitivity against antibiotics, millions of years of evolution have rendered the organellar ribosomes strikingly different from their ancestors. This is especially evident from their structure, because mitochondrial ribosomes contain typically much more protein and less rRNA. Furthermore, mitochondrial ribosomes greatly differ between species. Whereas the protein–rRNA ratio of bacterial ribosomes is 1:2, mitochondrial ribosomes of *S. cerevisiae* and *S. pombe* denote a 1:1 ratio and the relative gain in protein mass is even more pronounced in mitochondrial ribosomes of mammals (2:1). The functional significance of this huge increase in protein content is not entirely clear, but a likely explanation is that these ribosomes require additional stabilization due to the greatly reduced ribosomal RNA sequences and losses in structurally important RNA folds (Mears et al. 2006).

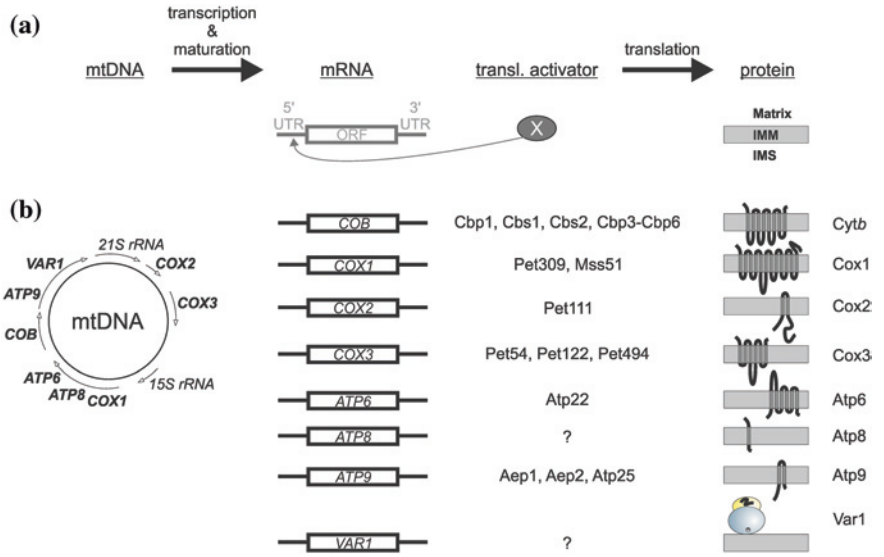

Fig. 5.1 Translational activators in yeast mitochondria. **a** General scheme of gene expression in yeast mitochondria. The mRNAs contain 5′- and 3′-untranslated regions (UTRs) flanking the respective open reading frame (ORF). Especially, the 5′-UTR is the target of specific translational activators (X), whose action is required to activate translation of their client mRNA. **b** Specific expression of genes encoded in the mitochondrial DNA with the help of translational activators. The mitochondrial genome (mtDNA) of *S. cerevisiae* encodes two ribosomal RNAs (*15S* and *21S* rRNA), eight proteins and 24 tRNAs (not shown). So far, translational activators for six of the eight mRNAs have been identified. The topologies of the mitochondrially encoded proteins in the inner membrane are depicted. Var1 is a component of the small ribosomal subunit. IMM, inner mitochondrial membrane. IMS, intermembrane space

The general process of protein synthesis in mitochondria involves many conserved translation factors and has mainly been analyzed by studying mammalian mitochondria, see also the Chap. 2. Whereas the elongation cycle of translation in mitochondria is assumed to closely resemble the bacterial process, termination of protein synthesis deviates from the ancestral system (Chrzanowska-Lightowlers et al. 2011; Kehrein et al. 2013). The initiation step of mitochondrial translation is only poorly understood. Unlike bacterial mRNAs or transcripts in the cytosol of eukaryotes, mitochondrial mRNAs do not contain Shine–Dalgarno sequences or 5′-cap structures that promote initiation of translation in these systems. Although in yeast nucleotides in the 15S rRNA of the small ribosomal subunit are complementary to sequences in mitochondrial mRNAs (Li et al. 1982), those regions do not fulfill a Shine–Dalgarno-like function as they are dispensable for translation (Costanzo and Fox 1988; Mittelmeier and Dieckmann 1995). Rather, sequences adjacent to the start AUG in the mRNA are implicated in this process. Accordingly, removal of the start AUG did not allow initiation of translation at alternative AUG codons within downstream sequences; changing the

start AUG to AUA in *COX2* and *COX3* mRNAs only modestly reduced synthesis of Cox2 and Cox3 (Folley and Fox 1991; Mulero and Fox 1994; Bonnefoy and Fox 2000). Importantly, the 5′- and 3′-untranslated regions (UTRs) are, at least in yeast, the target of transcript-specific translational activators. These factors are mediating in a yet unknown manner the translation of their specific client mRNA.

5.1.3 The Concept of Translational Activators and Their Possible Functions

Translational activators (TAs) in yeast mitochondria have been studied for more than four decades (Fig. 5.1). The cytochrome oxidase subunits Cox2 and Cox3 were the first examples for which the concept of specific translational activation in yeast mitochondria was introduced. Early studies showed that deletion of the nuclear genes *PET111* and *PET494* specifically impairs the expression of the mitochondrial *COX2* and *COX3* gene, respectively (Cabral and Schatz 1978). In subsequent years, genetic screens revealed that mutants lacking one TA (and therefore one mitochondrially encoded protein) could regain respiratory growth by remodeling of sequences within the mitochondrial genome. Those rearrangements led to the generation of fusion genes or the exchange of regulatory regions with the result that affected transcripts acquired 5′-UTRs of other genes, making their expression independent from the authentic, missing TA but dependent on the factor controlling synthesis of the other gene (Muller et al. 1984). In yeast, expression of six of the eight mitochondrially encoded proteins depends on TAs; these factors are described below.

How exactly TAs exert their function in translation is not yet understood. Different molecular functions have been suggested, and because there is experimental evidence for all of them, TAs might not share one universal function but rather exert their roles at different steps of the translation process and in some cases have more than one function. Some TAs like Cbp1, Pet309, Pet111, Aep2, and Atp25 are required for stabilizing the transcripts they act on (Poutre and Fox 1987; Payne et al. 1991; Manthey and McEwen 1995; Ellis et al. 1999; Islas-Osuna et al. 2002; Zeng et al. 2008).

Another possibility is that TAs work by supporting initiation of translation by, e.g., assisting to load the mRNA correctly onto the ribosome as Shine–Dalgarno-like sequences are absent in mitochondrial messengers. Interactions of TAs with both the 5′-UTR and mitochondrial ribosomes would support such an idea and have been shown primarily by genetic study (McMullin et al. 1990; Haffter et al. 1991; Haffter and Fox 1992; Fox 1996). A direct binding of TAs to sequences within specific 5′-UTRs has been demonstrated in the cases of Pet111 and Pet122, where substitutions of amino acids in the TA could rescue adverse mutations in the 5′-UTR of *COX2* and *COX3*, respectively (Costanzo and Fox 1993; Mulero and Fox 1993a).

Translational activators participate in the organization of mitochondrial protein synthesis. Many TAs are peripheral or integral membrane proteins, and therefore localize the mRNAs to the matrix face of the inner membrane to facilitate interactions with the permanently membrane-associated translation machinery in mitochondria (McMullin and Fox 1993; Sanchirico et al. 1998). However, the organization of translation by TAs might be even more intricate than this. For example, Pet309 was found to be present in a complex of a molecular mass of about 900 kDa that also contained Cbp1, a protein required to stabilize the cytochrome *b* mRNA (Krause et al. 2004). Pet309 was independently shown to be in contact with the general mRNA metabolism factor Nam1 (Naithani et al. 2003). A third study revealed a Nam1 interaction with the yeast mitochondrial RNA polymerase (Rodeheffer et al. 2001). From all these findings, a model was suggested that links transcription, mRNA maturation, and protection as well as translation at the inner mitochondrial membrane (Krause et al. 2004). Furthermore, the specific TAs of Cox1, Cox2, and Cox3 interact with each other and thereby organize expression of the three cytochrome oxidase subunits in a way that allows efficient assembly of this respiratory chain complex (Naithani et al. 2003).

The last, and comparably well documented, function of TAs is the regulation of mitochondrial protein synthesis in response to the efficiency of respiratory chain assembly. Respiratory chain complexes and the ATP synthase are composed of subunits produced by two different genetic systems and the assembly of these complex machineries is a highly intricate event. To allow efficient assembly of the respiratory chain, the expression of mitochondrially and nuclear encoded subunits has to be coordinated. Translational activators participate in these regulatory circuits in the case of the bc_1 complex, cytochrome oxidase, and the ATP synthase in yeast. In general, when assembly of an OXPHOS complex is blocked due to missing nuclear-encoded structural subunits or the absence of specific assembly factors, sequestration of a TA in an assembly intermediate that contains a mitochondrially encoded subunit of the OXPHOS complex lowers the amounts of the TA available to stimulate translation (Fig. 5.2). By this, mitochondrial protein synthesis is adjusted to levels that can successfully be assembled into OXPHOS complexes. The coupling of synthesis and assembly is a conserved process as it had first been described for the biogenesis of chloroplast photosystems and there been termed “control of epistatic synthesis” (CES) (Wollman et al. 1999). The detailed mechanisms of how specific TAs act in such regulatory feedback loops in yeast mitochondria are outlined in the last section of this chapter.

5.1.4 Synthesis of Cytochrome b

Cytochrome *b* is the only subunit of the bc_1 complex which is encoded in the mitochondrial genome and translation of its mRNA (*COB* mRNA) is dependent on several factors (Rödel 1997). The *COB* gene is co-transcribed with an adjacent tRNA and the resulting bi-cistronic precursor has to be processed in a complex way (Christianson et al. 1983; Hollingsworth and Martin 1986; Chen and

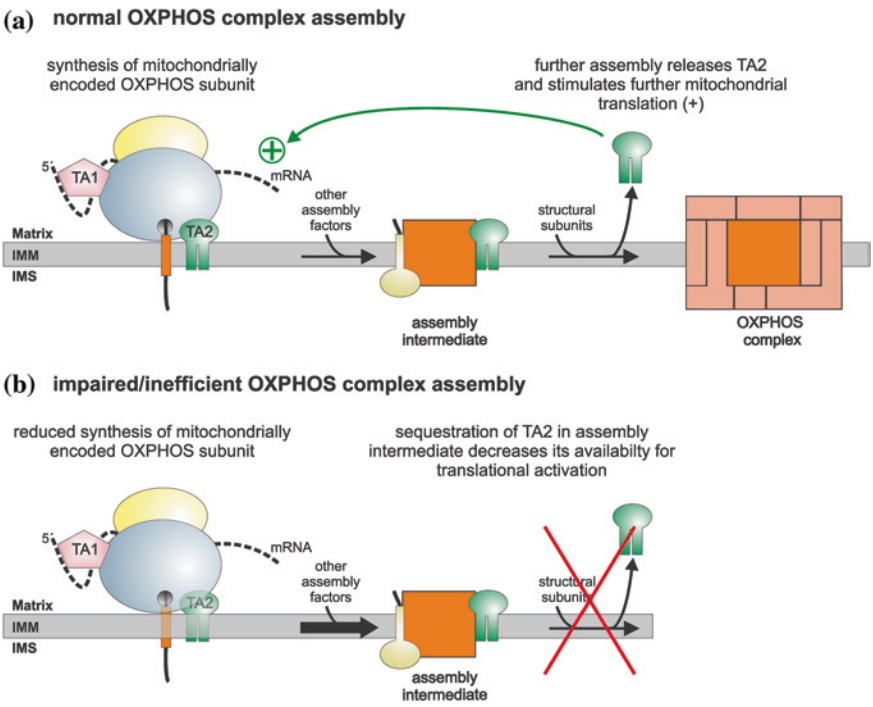

Fig. 5.2 The general principle of feedback loops regulating mitochondrial protein synthesis in response to the efficiency of OXPHOS complex assembly. Translation in mitochondria requires specific translational activators (TAs). Some TAs (TA2 in the scheme) have a dual role in activating translation of their client mRNA and in mediating assembly of the encoded protein into a respiratory chain complex. **a** Under normal circumstances when assembly is not disturbed, the TA2 is only transiently present in an assembly intermediate and released upon further assembly. It can then stimulate further translation of the regulated subunit (*dark orange*) at the mitochondrial ribosome (+). **b** If complex assembly is perturbed due to the absence of structural OXPHOS subunits or assembly factors encoded in the nucleus, TA2 cannot be released efficiently and is sequestered in the assembly intermediate. The TA is therefore not available for activating translation, which reduces of synthesis of the mitochondrially encoded OXPHOS subunit. By this, mitochondrial translation is adjusted to level that can be incorporated into OXPHOS complexes. IMM, inner mitochondrial membrane. IMS, intermembrane space

Martin 1988) The *COB* gene furthermore contains introns, some of which encode maturases that are required for the excision of introns within the same or other transcripts (Lazowska et al. 1980; Nobrega and Tzagoloff 1980; Dhawale et al. 1981; De La Salle et al. 1982). Both the unprocessed and the mature *COB* transcripts are shielded from exonucleolytic degradation by the factor Cbp1 (Weber and Dieckmann 1990; Mittelmeier and Dieckmann 1995; Islas-Osuna et al. 2002). This mRNA stabilization involves the interaction of Cbp1 with the 5′-UTR of cytochrome *b;* a single CCG triplet near the 5′-end is especially important (Chen and Dieckmann 1997). In addition to stabilization, Cbp1 is also directly necessary for translation of the *COB* mRNA (Islas-Osuna et al. 2002).

Four other TAs are specifically required for the synthesis of cytochrome *b*. Yeast cells lacking either the product of the *CBS1* or the *CBS2* gene cannot translate *COB* mRNA and accumulate the unprocessed pre-*COB* transcript (Rödel et al. 1985; Rödel 1986). Because Cbs1 and Cbs2 are needed for cytochrome *b* synthesis also in a strain where the *COB* gene does not contain any introns, they do not function in processing of pre-*COB* (Muroff and Tzagoloff 1990). The accumulation of the unprocessed transcript is presumably a secondary effect in Δ*cbs1* and Δ*cbs2* cells, because synthesis of the maturases encoded within introns requires translation of the pretranscript. Similar to the case of Cbp1, the *COB* 5′-UTR dictates the dependence on Cbs1 and Cbs2 (Rödel et al. 1985; Rödel and Fox 1987). The region within the 5′-UTR recognized by the two TAs lies in the sequence −232 to −4 relative to the start AUG at +1 (Mittelmeier and Dieckmann 1995). However, a direct interaction between either Cbs1 or Cbs2 and the 5′-untranslated region of *COB* mRNA was not yet shown, so it is not clear whether the factors act directly or indirectly through yet unknown components.

Additional factors involved in cytochrome *b* translation are the proteins Cbp3 and Cbp6, which form a functionally and structurally inseparable complex that binds to mitochondrial ribosomes in close proximity to the tunnel exit (Gruschke et al. 2011). In contrast to Δ*cbs1* and Δ*cbs2* cells, the pre-*COB* transcript is processed and matured similar to the wild type in the absence of Cbp3 or Cbp6 (Dieckmann and Tzagoloff 1985; Gruschke et al. 2011). Despite this, cytochrome *b* cannot accumulate in the mutants and the phenotype cannot be suppressed by a typical gene rearrangement within the mitochondrial genome (Tzagoloff et al. 1988; Gruschke et al. 2012; Kühl et al. 2012). This can be attributed to the fact that the Cbp3–Cbp6 complex exerts a second function in cytochrome *b* biogenesis; it is required for the stabilization and assembly of the newly synthesized protein. As soon as cytochrome *b* is fully synthesized, Cbp3–Cbp6 binds to the protein, the complex is released from the ribosome and after recruitment of the assembly factor Cbp4 cytochrome *b* is fed into the bc_1 complex assembly line (Gruschke et al. 2011; Gruschke et al. 2012). This dual role of Cbp3–Cbp6 in both translation of the *COB* mRNA and assembly of cytochrome *b* enables Cbp3–Cbp6 to act in a regulatory feedback circuit that adjusts the level of cytochrome *b* synthesis to the assembly efficiency of the bc_1 complex (Gruschke et al. 2012). This regulation is explained in more detail in the last section of the chapter.

5.1.5 Synthesis of Mitochondrially Encoded Cytochrome Oxidase Subunits

Three subunits of cytochrome oxidase (COX, complex IV) are encoded in the mitochondrial genome, namely Cox1, Cox2, and Cox3. The TA for *COX2*, Pet111, and one of the TAs for *COX3*, Pet494, served as the first examples for specific translational activation in mitochondria (Cabral and Schatz 1978). Due to the pioneering work of Tom Fox, Pet111 is the best-studied translational activator.

Like for other TAs, the phenotype of the *PET111* deletion mutant was found to be suppressible by the exchange of *COX2* regulatory regions to 5′-UTRs of other mitochondrial genes (Poutre and Fox 1987; Mulero and Fox 1993b). The 5′-UTR of the *COX2* mRNA is relatively short (54 bases), making mutagenetic analyses spanning the complete sequence easier than in other cases. By this means, it was shown that the sequence between −16 and −47 (relative to the translation start at +1) is sufficient to confer Pet111-mediated translational activation; within this region lies a predicted stem–loop structure between position −20 and −35 that is especially important (Dunstan et al. 1997). Like in the case of Cbp1 and the *COB* 5′-UTR, a direct interaction between this structural motif in the *COX2* 5′-UTR and Pet111 is very likely, because mutations in the protein can rescue base exchanges in the mRNA (Mulero and Fox 1993a). However, the functional role of Pet111 in translational activation of *COX2* on the molecular level has, as for the other TAs, not been elucidated yet.

Translational activation of *COX3* mRNA depends on three proteins, Pet54, Pet122, and Pet494 (Cabral and Schatz 1978; Muller et al. 1984; Costanzo and Fox 1986, 1988). All of these factors act on the 613 nucleotide long 5′-UTR of *COX3* mRNA (Costanzo and Fox 1988). In the case of Pet122, this interaction presumably is directed as a mutation within the protein can restore translation of an mRNA lacking a functionally important part of the 5′-UTR (Costanzo and Fox 1993). Furthermore, Pet54, Pet122, and Pet494 interact with each other at the inner mitochondrial membrane and thereby presumably help localizing synthesis of this cytochrome oxidase subunit to the membrane (McMullin and Fox 1993; Brown et al. 1994). In addition to a function in Cox3 synthesis, Pet54 has been proposed to play a role in maturation of the *COX1* mRNA (Valencik and McEwen 1991).

The third cytochrome oxidase subunit encoded in the mitochondrial genome is Cox1. Its synthesis depends on two proteins that are involved in posttranscriptional processes. Pet309 is required for translation of the *COX1* transcript, because yeast strains harboring an intronless *COX1* gene accumulate the mature mRNA, but fail to synthesize Cox1 (Manthey and McEwen 1995). Pet309 belongs to the class of PPR proteins that contain pentatricopeptide repeats, a motif involved in protein-RNA interactions (Lipinski et al. 2011). All of the seven PPRs of Pet309 are required for supporting translation of the *COX1* mRNA, suggesting direct interaction of the protein with the 5′-UTR of the messenger (Tavares-Carreon et al. 2008). Mss51 is the second protein involved in translation of the *COX1* transcript. Although initially thought to be required for splicing of the *COX1* precursor mRNA, experiments with strains harboring an intronless *COX1* gene showed that Mss51 rather functions as a translational activator (Faye and Simon 1983; Decoster et al. 1990). The *COX1* 5′-UTR again was shown to direct Mss51 dependence; however, the exchange of this regulatory region for that of another mitochondrial gene did not bypass the requirement for Mss51 (Perez-Martinez et al. 2003; Zambrano et al. 2007). The reason for this is a second posttranslational function of Mss51 in Cox1 biogenesis; it interacts with newly synthesized Cox1 and is part of cytochrome oxidase assembly intermediates (Perez-Martinez

et al. 2003; Barrientos et al. 2004). Recent studies have shown that the synthesis of Cox1 is regulated in a highly complex manner in response to cytochrome oxidase assembly. In this process, Mss51 plays a key role by mediating both translation of the *COX1* mRNA and assembly of this respiratory chain complex (Fontanesi et al. 2008; Mick et al. 2011). It thus represents a second example of a TA mediating feedback modulation of mitochondrial protein synthesis in the context of OXPHOS complex assembly (see below).

5.1.6 Synthesis of Mitochondrially Encoded ATP Synthase Subunits

The mitochondrial genome of *S. cerevisiae* contributes three subunits to the formation of the ATP synthase, Atp6, Atp8 and Atp9, all of which are part of the membrane-integrated F_o unit. The *ATP6* and *ATP8* genes are transcribed as one long precursor mRNA together with *COX1* (Simon and Faye 1984). After endonucleolytic cleavage of this pretranscript, maturation, and stabilization of the *ATP8/ATP6* bi-cistronic and/or the single mRNAs is accomplished by several nuclear encoded factors: Nca2, Nca3, and Nam1 (which is not only specific for Atp6 and Atp8) and Aep3 (Groudinsky et al. 1993; Camougrand et al. 1995; Pelissier et al. 1995; Ellis et al. 2004).

Translation of *ATP6* depends on the factor Atp22 (Zeng et al. 2007). Similar to other TAs, the absence of Atp22 can be overcome by a mitochondrial gene rearrangement leading to the generation of a *Cox1::ATP6* transcript. Translation of this mRNA is only dependent on Pet309 and Mss51, the TAs of *COX1*, but not on Atp22 (Zeng et al. 2007). In accordance, efficient synthesis of a mitochondrially encoded reporter gene was strictly dependent on the presence of Atp22 (Rak and Tzagoloff 2009). Although *ATP6* and *ATP8* are produced from a bi-cistronic transcript, *ATP22* deletion mutants specifically lack Atp6 but show normal translation rates of *ATP8* (Zeng et al. 2007). This suggests that a translational activator for *ATP8* still awaits identification.

The core component of the F_o part of the ATP synthase is an oligomer of Atp9 subunits that forms the proton conducting channel. The *ATP9* gene is transcribed together with an adjacent tRNA and the *VAR1* gene and the polycistronic transcript is matured by endonucleolytic cleavage (Zassenhaus et al. 1984). The importance of the 5′-UTR of *ATP9* was recognized very early, as insertion of bases into this region impaired translation (Ooi et al. 1987). Three proteins influence translation of the *ATP9* mRNA: Aep1, which acts as a TA, Aep2 that is either required for the stabilization of the *ATP9* transcript or stimulating its translation and Atp25, which has a dual role in translation and assembly of ATP synthase (Payne et al. 1991, 1993; Ellis et al. 1999; Zeng et al. 2008). Atp25 is split into two halves and both portions function in mitochondria. The C-terminal half of the protein is conferring stability to the *ATP9* mRNA and expression of this part of the protein is sufficient to allow Atp9 synthesis in the *ATP25* deletion mutant

(Zeng et al. 2008). In the absence of the N-terminal half of Atp25; however, the translated Atp9 is not stably assembled into the Atp9-oligomer. This suggests that the N-terminal half of Atp25 is not dispensable for the biogenesis of Atp9 and might even mediate assembly of the Atp9 ring. Hence, Atp25 is a protein of dual function with a probability to modulate expression of *ATP9* in a feedback loop. However, this hypothesis has not yet been analyzed experimentally. Importantly, it was recently demonstrated that the synthesis of Atp6 and Atp8 is regulated in response to the assembly process of the ATP synthase complex (Rak and Tzagoloff 2009). This is described in detail below.

5.2 Nuclear Control of Protein Synthesis in Yeast Mitochondria

Translational activators are encoded in the nucleus. Soon after the discovery of Pet111 and Pet494 and the establishment of the concept of specific translational activation, regulation/control of the expression of these nuclear genes was investigated. Studies using a yeast strain with a chromosomal gene fusion consisting of the *COX3*-specific *PET494* and the *E. coli* β-galactosidase gene *lacZ* revealed that Pet494 is expressed at very low levels (Marykwas and Fox 1989). The TAs Pet122 and Pet111 are present in similarly low amounts (Fox 1996). The low abundance of translational activators implies that they are rate limiting for mitochondrial protein synthesis. This was confirmed for Pet494 by investigating diploid yeast strains homo- or heterozygous for the *PET494* locus or haploids carrying a high copy plasmid to overexpress the gene. Additionally, these strains harbored a mitochondrial genome that encodes the reporter construct *ARG8* ^{m} (Steele et al. 1996). *ARG8* is a nuclear gene coding for a soluble enzyme, which is normally posttranslationally imported into mitochondria and involved in the biogenesis of arginine. The recoded version of the gene *ARG8* ^{m} was integrated into the mtDNA of yeast deficient in the nuclear copy of *ARG8*. In the study of Steele et al., the open reading frame of *COX3* was substituted by the *ARG8* ^{m} gene (*cox3::ARG8* ^{m} mtDNA), making Arg8 synthesis dependent on *COX3*-specific translational activation. The available amount of Pet494 clearly correlated with the Arg8 expression rate, while *cox3::ARG8* ^{m} expression only moderately correlated with Pet122 level (Steele et al. 1996).

The expression of many genes involved in respiration in yeast is modulated over a wide range of growth conditions and TAs seem to be no exception to this. *PET494* expression is subject to catabolite repression (Marykwas and Fox 1989). In the presence of glucose, the levels of the TA drop four to sixfold in comparison to cells grown on nonfermentable carbon sources. Furthermore, synthesis of Pet494 is regulated by oxygen, but in contrast to the transcriptional repression by glucose this is rather achieved on a translational level (Marykwas and Fox 1989). Interestingly, expression of *PET494* is heme independent. This is opposed to other respiratory genes that are responding to oxygen levels, where transcriptional

upregulation under aerobic conditions is mediated by heme (Guarente and Mason 1983; Keng and Guarente 1987). The regulation of the expression of other TAs was not analyzed similarly detailed. However, expression of many genes necessary for respiration and especially subunits of the OXPHOS system is influenced by growth conditions (Guarente and Mason 1983; Lowry et al. 1983; Myers et al. 1987; Forsburg and Guarente 1989). Taken into account the feedback regulatory circles that were revealed in the last years, this regulation of nuclear gene expression can be considered as an example of how mitochondrial gene expression is influenced by carbon source, oxygen levels, or presence of heme .

5.3 Regulation of Mitochondrial Protein Synthesis in Response to Assembly of the Oxphos System

Both the nuclear as well as the mitochondrial protein synthesis machinery contribute subunits to the OXPHOS complexes. To ensure efficient assembly, these expression systems have to be coordinated temporally and spatially in a precise manner. In recent years, different groups have revealed how regulation of mitochondrial protein synthesis is accomplished and how the levels of mitochondrially encoded subunits are adjusted to allow an efficient OXPHOS assembly process (Fig. 5.2). The general principle is that TAs with dual functions are sequestered in OXPHOS assembly intermediates. When assembly proceeds normally, the TA is released to stimulate synthesis of its client protein. In contrast, when further assembly fails, the TA is trapped in the assembly intermediate and not available to activate new rounds of translation.

5.3.1 Regulation of Cytochrome b Synthesis

The yeast bc_1 complex is composed of nine nuclear-encoded subunits that are assembled around the core component cytochrome *b*, which is produced by the mitochondrial genetic system. Catalytically active are only the three proteins, cytochrome *b*, cytochrome c_1 (Cyt1), and the Fe/S protein Rip1, whereas the remaining seven subunits are accessory structural subunits. The step-wise assembly process involves four intermediates and has mainly been analyzed by the use of yeast strains lacking individual structural subunits of the bc_1 complex and their analysis by Blue Native polyacrylamide gel electrophoresis (BN PAGE) (Zara et al. 2007, 2009a, b; Gruschke et al. 2012; Smith et al. 2012). Assembly starts with synthesis and membrane insertion of cytochrome *b*, which is immediately bound by the Cbp3–Cbp6 complex (Fig. 5.3). Recruitment of the assembly factor Cbp4 results in assembly intermediate I that serves as a pool of unassembled cytochrome *b* even at steady state. Addition of the first two nuclear-encoded

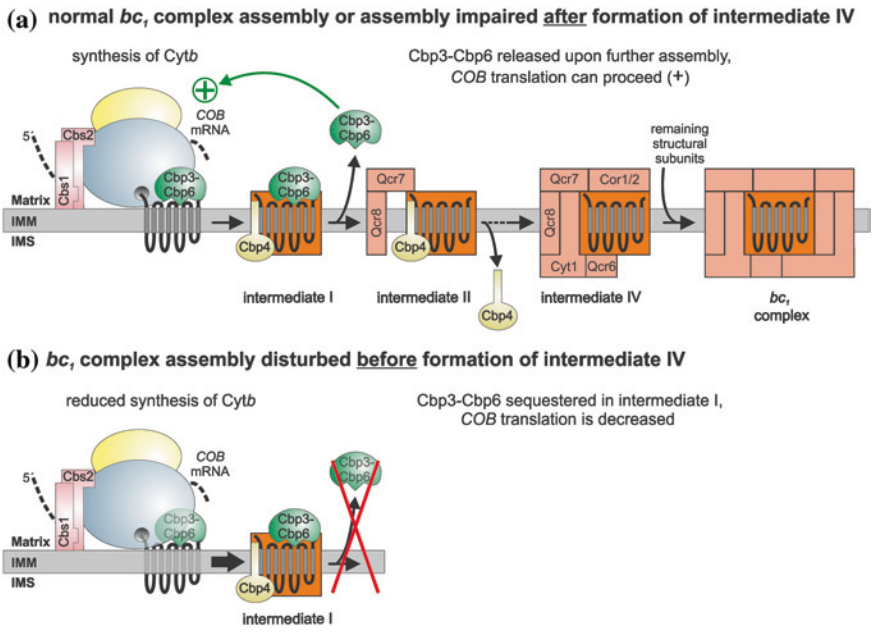

Fig. 5.3 Schematic representation of the regulatory feedback loop modulating cytochrome *b* synthesis in response to the assembly of the bc_1 complex. The Cbp3–Cbp6 complex exerts a dual role in the biogenesis of cytochrome *b*: In its ribosome-bound form it acts as a translational activator and together with Cbs1 and Cbs2 stimulates translation of the *COB* mRNA. It is also present as a nonribosome-bound form in association with cytochrome *b* and Cbp4, forming the first assembly intermediate of the bc_1 complex assembly line. A: Cytochrome *b* assembles through four intermediates into a functional bc_1 complex, three of which are depicted in the scheme. When assembly is undisturbed or can proceed at least until intermediate IV is formed, Cbp3–Cbp6 is released from intermediate I upon further assembly, can again activate *COB* mRNA translation at the ribosome (+) and cytochrome *b* synthesis is not affected. B: If complex assembly is disturbed before intermediate IV is formed, Cbp3–Cbp6 cannot be released efficiently and is sequestered in the accumulating intermediate I (*thick black arrow*). The complex is therefore not available for activating translation and consequently cytochrome *b* synthesis is reduced. IMM, inner mitochondrial membrane. IMS, intermembrane space

structural subunits Qcr7 and Qcr8 induces release of Cbp3–Cbp6, whereas Cbp4 stays attached. The cytochrome *b*-Cbp4-Qcr7-Qcr8 complex represents the second assembly intermediate and is further joined by the two core proteins Cor1 and Cor2. Addition of Cyt1 and the small acidic accessory subunit Qcr6 to intermediate III forms intermediate IV, which was previously described as the 500 kDa complex (Zara et al. 2009b). The incorporation of two accessory subunits (Qcr9 and Qcr10) and the last catalytic subunit (Rip1) completes formation of the bc_1 complex. When assembly is disturbed before intermediate IV can be generated, the synthesis of cytochrome *b* is reduced (Gruschke et al. 2012) (Fig. 5.3). This is caused by sequestration of Cbp3–Cbp6 in assembly intermediate I, which accumulates under these conditions and as a result the Cbp3–Cbp6 complex is not

available at the mitochondrial ribosome to fulfill its function as a TA for the *COB* mRNA (Gruschke et al. 2012). This negative feedback can be overcome by overexpression of the Cbp3–Cbp6 complex, demonstrating its key role in this process. Formation of assembly intermediate IV seems to be a critical point in the pathway as disturbance of the last assembly step by either deletion of one of the structural subunits *QCR9*, *QCR10*, *RIP1* or required assembly factors (*MZM1*, *BCS1*) does not lead to reduced cytochrome *b* translation.

5.3.2 Regulation of Cox1 Synthesis

The cytochrome oxidase (COX, complex IV) is composed of 11 subunits in yeast, three of which are encoded in the mitochondrial genome. Two of these three subunits harbor redox-active heme and/or copper co-factors. Electrons flow from the Cu_A center of Cox2 to the heme *a* cofactor of Cox1, from where they are passed further to the active site of Cox1 composed of the Cu_B center and heme a_3. Heme a_3 binds molecular oxygen which serves as the final electron acceptor. The assembly of this OXPHOS complex is characterized very well and assisted by a considerable number of factors involved in co-factor acquisition, mediation of subunit interaction and feedback regulation (Fontanesi et al. 2006; Mick et al. 2011). Assembly of cytochrome oxidase is initiated from the central subunit of the complex, Cox1. Unassembled Cox1 with its redox-active cofactors is potentially harmful for cells as it may give rise to reactive oxygen species (Khalimonchuk et al. 2007). To ensure integrity of the cell, Cox1 synthesis has to be monitored precisely and adjusted to levels that can successfully be incorporated into COX. In recent years, it was found that Cox1 translation in yeast is subject to a complex feedback regulatory circle that achieves this fine tuning (Mick et al. 2011). The key role in this feedback loop is played by the dually functioning protein Mss51, which acts as a TA for *COX1* mRNA as well as a Cox1-assembly factor by binding the newly synthesized protein (Perez-Martinez et al. 2003). Like Cbp3–Cbp6 in the case of the bc_1 complex, Mss51 is sequestered in assembly intermediates that cannot be resolved when further assembly is blocked and thereby is precluded from activating new rounds of *COX1* translation (Fig. 5.4). Very recently, it was demonstrated that Mss51 contains two heme binding motifs in its N-terminus, thereby allowing it to act as a heme sensor and coordinate COX assembly with heme availability (Soto et al. 2012).

Besides Mss51, several other factors participate in COX assembly and the feedback regulation mechanism (Fig. 5.4). The presence of Cox14 and Coa3/Cox25 is required to allow efficient interaction of Mss51 with newly synthesized Cox1, and thus they act as negative regulators of *COX1* synthesis by ensuring efficient sequestration of Mss51 (Barrientos et al. 2004; Perez-Martinez et al. 2009; Mick et al. 2010; Fontanesi et al. 2011). *COX1* feedback regulation depends on the C-terminal region of Cox1 itself. Mutants lacking this part of the protein can synthesize and assemble Cox1 into a functional cytochrome oxidase, but do not exhibit assembly

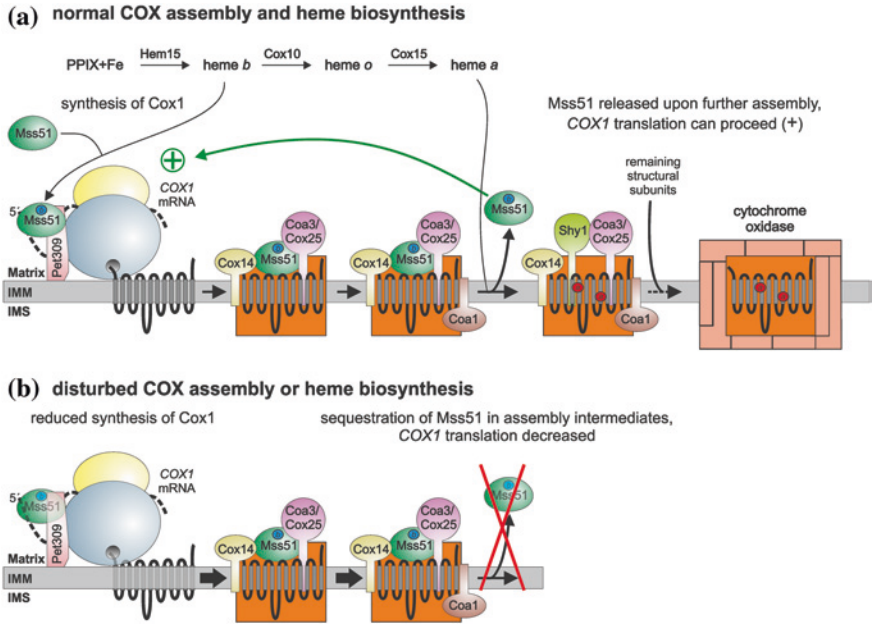

Fig. 5.4 Schematic representation of the regulatory feedback loop modulating Cox1 synthesis in response to the assembly of the COX complex. **a** Mss51 has two functions in the biogenesis of this OXPHOS complex; in concert with Pet309 it serves as a translational activator for *COX1* mRNA and, in addition, Mss51 is part of assembly intermediates, acting as a Cox1 chaperone. Mss51 is a heme-binding protein, which additionally allows to regulate *COX1* synthesis in response to the heme homeostasis of the cell. Hem15 (ferrochelatase) incorporates iron into the protophorphyrin IX ring (PPIX), thereby forming heme *b* from which subsequently heme *a*, one of the cofactors present in Cox1, is synthesized through the concerted action of Cox10 and Cox15. Together with hemylated Mss51, Cox14, Coa3/Cox25, and Coa1 are part of early Cox1 assembly intermediates. Presumably, at the step where Cox1 is hemylated and Shy1 enters the pathway, Mss51 is released and can again activate *COX1* mRNA translation (+). Incorporation of the remaining structural subunits releases the other assembly factors until formation of cytochrome oxidase is completed. **b** If heme biosynthesis or COX assembly is disturbed, Mss51 cannot function properly or is sequestered in assembly intermediates and therefore not available for activating translation; Cox1 synthesis consequently is reduced. IMM, inner mitochondrial membrane. IMS, intermembrane space

responsive reduction of Cox1 synthesis. It has been speculated that the molecular reason for this lies in the weakened interaction between Mss51 and Cox14 (Shingu-Vazquez et al. 2010). Coa1, Coa2, Shy1, and the mitochondrial Hsp70 chaperone Ssc1 are additional factors participating in COX assembly (Barrientos et al. 2002; Pierrel et al. 2007; Fontanesi et al. 2008; Pierrel et al. 2008; Fontanesi et al. 2010). The exact molecular composition of all COX assembly intermediates is, however, still under debate (McStay et al. 2012). A subcomplex consisting of Mss51, Cox14, Coa3/Cox25, and Coa1 bound to an oxidatively harmless, unhemylated form of Cox1 is stable in wild-type cells and presumably serves as a pool of assembly

competent Cox1 (Khalimonchuk et al. 2010). In contrast, the Shy1-containing assembly intermediate comprises hemylated Cox1; however, Shy1 is most likely not required for hemylation *per se*, but rather stabilizes Cox1 in a conformation allowing the insertion of heme a_3. Although it is not entirely resolved yet, Mss51 presumably is released from Cox1 when Shy1 enters the assembly pathway. Mss51 can then again act as a TA and induce further Cox1 synthesis.

5.3.3 Regulation of Atp6/8 Synthesis

The ATP synthase is composed of three functionally and structurally distinct parts. The membrane-embedded F_o part comprises Atp9 subunits, which form a ring-like structure, and the two proteins Atp6 and Atp8. These three proteins are encoded in the mitochondrial genome. The hydrophilic F_1 part is formed by a hexamer of alternating α and β subunits that mediate ATP synthesis and the central stalk, which is made up of subunits γ, δ, and ε. The stalk is in contact to the Atp9-ring as well as the $\alpha_3\beta_3$ hexamer. The third part of the enzyme is the peripheral stator stalk made up of four subunits, which is attached to both the $\alpha_3\beta_3$ oligomer and Atp6 in the membrane. By this, the $\alpha_3\beta_3$ hexamer, Atp6, and the stator form the stationary part of the enzyme. Driven by the electrochemical gradient across the membrane, protons flow back from the intermembrane space into the matrix at the interface between the Atp9 ring and Atp6, thereby rotating the Atp9 part and the central stalk stepwise and inducing conformational changes at the catalytic sites of the $\alpha_3\beta_3$ hexamer that drive ATP synthesis (Stock et al. 2000). The assembly process of the ATP synthase is not understood in every detail (Ackerman and Tzagoloff 2005; Rak et al. 2009). Early experiments indicated that assembly of the F_1 unit is independent from assembly of F_o (Schatz 1968). The current idea is that ATP synthase assembly involves to distinct, but coordinately formed modules which are joined at the end (Rak et al. 2011). The main F_o component, the Atp9 ring, is assembled from Atp9 monomers with the help of the N-terminal part of Atp25 and then interacts with the pre-assembled F_1 unit (Zeng et al. 2008; Rak et al. 2011). In parallel, a complex of Atp6, Atp8, and at least two stator stalk subunits is generated. Together with Atp6, the Atp9 ring forms the proton translocating channel of the enzyme complex. The joining of Atp6 with the Atp9 ring seems to occur at a rather late step of assembly and involves the assembly factor Atp10 and the inner membrane protein Oxa1 (Tzagoloff et al. 2004; Jia et al. 2007).

In 2009, Rak and Tzagoloff reported that translation of the *ATP8/ATP6* bi-cistronic mRNA is dependent on F_1 assembly (Fig. 5.5) (Rak and Tzagoloff 2009). Mutants lacking assembly factors required for the formation of the F_1 unit, Atp11 or Atp12, or the two main structural F_1 subunits α and β display reduced synthesis rates of Atp6 and Atp8. It was excluded that this was caused by an increased turnover of the newly translated proteins by analyzing expression of the *ARG8* ^{m} reporter genes (*atp6::ARG8* ^{m} or *atp8::ARG8* ^{m}) that revealed impaired Arg8 synthesis. Overexpression of Atp22, the translational activator of *ATP6*, was able to

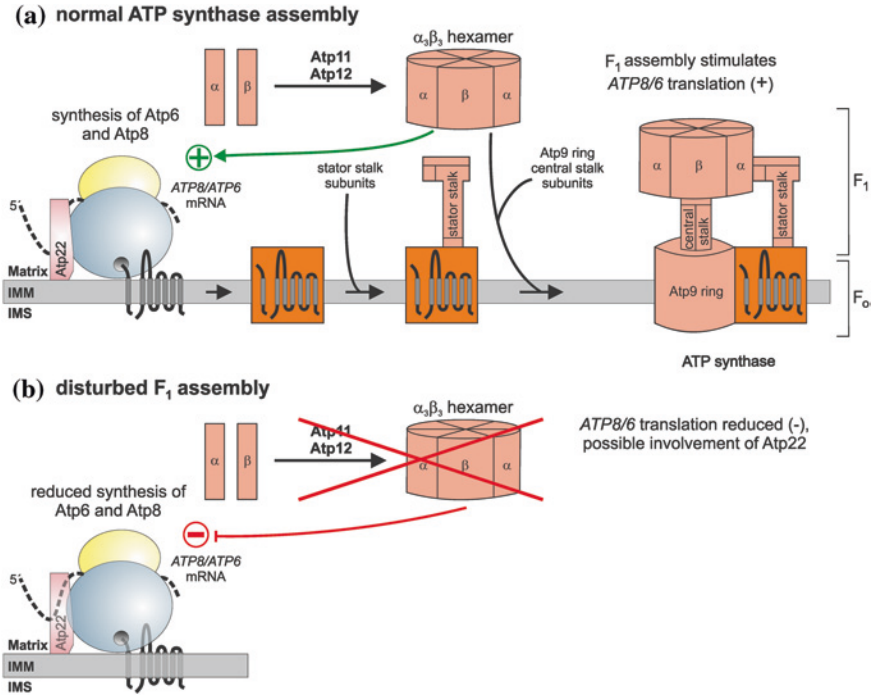

Fig. 5.5 Schematic representation of the regulatory feedback loop modulating Atp6/Atp8 synthesis in response to the assembly of the ATP synthase. A: The mitochondrially encoded ATP synthase subunits Atp6 and Atp8 are translated from a bi-cistronic mRNA with the help of the translational activator for *ATP6*, Atp22, and a yet unknown translational activator for *ATP8*. Atp6 and Atp8 are after their synthesis assembled with the stator stalk. The monomeric forms of the F_1 subunits α and β are prevented from aggregation and assembled into the $\alpha_3\beta_3$ hexamer by the assembly factors Atp11 and Atp12. After addition of the central stalk subunits, the F_1 part is joined to the Atp9 ring and the Atp6/8 module, forming the fully assembled ATP synthase. The successful assembly of the $\alpha_3\beta_3$ hexamer is required for efficient translation of the *ATP8/ATP6* mRNA (+). B: If F_1 formation is perturbed, synthesis of Atp6 and Atp8 is impaired (−). This regulation presumably involves Atp22, for details see text. IMM, inner mitochondrial membrane. IMS, intermembrane space

suppress the phenotype (Rak and Tzagoloff 2009). Currently, it is not clear how exactly this feedback regulation is mediated. It could be achieved either by the sequestration of Atp22 in some form of assembly intermediate (similar to the cases of Cbp3–Cbp6 and Mss51) or it could involve yet uncharacterized components. This system is physiological important, as it prevents the dissipation of the membrane potential in case the F_o part cannot efficiently be coupled to the F_1 complex. Although the F_1-dependent regulation of F_o biogenesis mechanistically differs from the feedback-regulated expression of *COB* or *COX1*, it provides another example of how mitochondrial translation is adjusted to the level of cytoplasmic protein synthesis.

5.4 Outlook

Biogenesis of OXPHOS complexes of dual genetic origin requires cross-talk of the two genetic systems involved. This regulation occurs at the level of mitochondrial protein synthesis, which is modulated by TAs that sense efficiency of assembly to down-regulate expression of their client protein when assembly fails. Despite the fact that specific translational activation of mitochondrial protein synthesis by nuclear genes is known since more than 40 years, we do not yet understand which exact molecular functions TAs exert during protein synthesis in the organelle. In addition to a presumably direct role in translation, mitochondrial TAs appear to be implicated in the organization of translation. The organization of cytochrome *b* biogenesis might serve as a good example to illustrate this: Cytochrome *b* is only efficiently synthesized when one of its TAs, the Cbp3–Cbp6 complex is present at the ribosomal tunnel exit (Gruschke et al. 2011). Because Cbp3–Cbp6 is also an essential assembly factor for cytochrome *b*, it is ensured that the newly synthesized protein experiences an optimally tailored environment for further assembly. Indeed, when cytochrome *b* is synthesized from an mRNA containing the 5′-UTR of another transcript, the proteins fails to accumulate robustly, while rates of synthesis of this ectopically expressed protein are indistinguishable from the authentic protein (Gruschke et al. 2012). Similar observations have also been reported previously for other ectopically expressed proteins (Sanchirico et al. 1998), suggesting that in mitochondria, each mRNA is translated by ribosomes that are specifically designed to optimally support biogenesis of the client protein (Gruschke and Ott 2010). It thus appears that mitochondrial protein synthesis is probably much more sophisticated organized than anticipated and that this system still harbors many exciting previously unidentified features.

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