The interactome of the yeast mitochondrial ribosome
Organization of mitochondrial post-transcriptional regulation, membrane protein insertion and quality control

Braulio Vargas Möller-Hergt

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Abstract
The proteins found in mitochondria originate from two different genetic systems. Most mitochondrial proteins are synthesized in the cytosol and post-translationally imported into the organelle. However, a small subset of mitochondrial proteins is encoded in an organelle-resident genome. Mitochondria contain factors responsible for replication, transcription and, most important for this thesis, synthesis of the mitochondrially encoded proteins. In the course of evolution the mitochondria specific ribosomes were extensively remodeled. The reasons for many of these adaptations are currently not well understood. For example, the mitoribosome is less stable and abundant than its bacterial counterpart. Therefore, I contributed in the development of robust biochemical tools in order to isolate and analyze the intact yeast mitoribosome and interaction partners by mass spectrometry. The results revealed a higher order organization of mitochondrial gene expression in complexes that we termed MIOREX (mitochondrial organization of gene expression). Besides the mitoribosome, MIOREX complexes contain factors involved in all steps of gene expression. This study also established many new ribosomal interaction partners, among them some proteins that were previously completely uncharacterized. In order to study these proteins, I refined the mass spectrometry approach, allowing a subunit-specific assignment of ribosomal interaction partners. The Mrx15 protein was determined by this approach as an interactor of the large subunit. I established that Mrx15 has overlapping functions with the ribosome receptor Mba1. Both proteins are necessary for mitoribosome membrane attachment and co-translational Cox2 membrane insertion. In a subsequent study I found a functional interaction of MRX15 and MBA1 with the regulators of the membrane-bound AAA proteases of the mitochondrial quality control system. Furthermore, the absence of Mrx15 leads to increased proteotoxic stress resistance of yeast cells. These results demonstrate an interesting connection between the mitochondrial quality control membrane insertion machineries, suggesting an early quality control step during the biogenesis of mitochondrially encoded proteins. In addition, we could reveal a subunit-specific interaction of translational activators and client mRNAs with the mitochondrial ribosome. This organization demonstrated how cytochrome b synthesis is pre-organized by specific translational activators independently of the COB mRNA. In summary, the work in this thesis showed how the vast and diverse interactome of the yeast mitoribosome organizes and regulates mitochondrial translation. These regulation mechanisms highlighted many organelle specific features. The work presented here will serve as starting point to design future studies aimed at a better understanding on how mitochondria adapted to organize gene expression inside the organelle.

Keywords: Mitochondria, mitochondrial post-transcriptional regulation, mitochondrial ribosome, membrane protein insertion, mitochondrial quality control.

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THE INTERACTOME OF THE YEAST MITOCHONDRIAL RIBOSOME

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Organization of mitochondrial post-transcriptional regulation, membrane protein insertion and quality control

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The way to get good ideas is to get lots of ideas and throw the bad ones away.

- Linus Pauling -
List of publications


Additional publications

### Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AAA</td>
<td>ATPases associated with various cellular activities</td>
</tr>
<tr>
<td>COB</td>
<td>Cytochrome $b$ (gene or mRNA)</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>ES</td>
<td>rRNA expansion segment</td>
</tr>
<tr>
<td>IMS</td>
<td>Intermembrane space</td>
</tr>
<tr>
<td>LRPPRC</td>
<td>Leucine-rich PPR motif-containing</td>
</tr>
<tr>
<td>LSU</td>
<td>Large ribosomal subunit</td>
</tr>
<tr>
<td>MIOREX</td>
<td>Mitochondrial organization of gene expression</td>
</tr>
<tr>
<td>OXPHOS</td>
<td>Oxidative phosphorylation</td>
</tr>
<tr>
<td>SRP</td>
<td>Signal recognition particle</td>
</tr>
<tr>
<td>SSU</td>
<td>Small ribosomal subunit</td>
</tr>
<tr>
<td>STED</td>
<td>Stimulated emission depletion</td>
</tr>
<tr>
<td>TACO1</td>
<td>Translational activator of Cox1</td>
</tr>
<tr>
<td>TIM</td>
<td>Translocase of the inner membrane</td>
</tr>
<tr>
<td>TOM</td>
<td>Translocase of the outer membrane</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
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Introduction

Mitochondria, a host for cellular energy metabolism

Mitochondria are the host for many catabolic biochemical processes. The TCA cycle, degradation of amino acids and the β-oxidation of fatty acids generate reduction equivalents in the form of NADH and FADH$_2$ in the organelle. These reduction equivalents are used to synthesize ATP by oxidative phosphorylation (OXPHOS). Mitochondria are compartmentalized by two lipid bilayers. The outer and inner mitochondrial membranes surround the matrix of the organelle (Figure 1A). The inner mitochondrial membrane houses the respiratory chain, which is responsible for OXPHOS. The OXPHOS system generally consists of four respiratory chain complexes and the ATP synthase: (a) the NADH dehydrogenase (complex I); (b) the succinate dehydrogenase (complex II); (c) the cytochrome $c$ reductase (complex III); (d) the cytochrome $c$ oxidase (complex IV); (e) the ATP synthase (complex V).

During OXPHOS electrons from NADH and FADH$_2$ oxidation are fed into the respiratory chain and employed to reduce molecular oxygen to water. The electrons are channeled through the different respiratory complexes by membrane-bound electron carriers in a series of exergonic redox reactions [1]. In complex I, III and IV these reactions are coupled to an endergonic transfer of protons across the inner mitochondrial membrane [2]. The energy from the generated proton gradient between the mitochondrial matrix and the intermembrane space is used by the ATP synthase to generate ATP from ADP and inorganic phosphate (Figure 1B). This process is the main source of chemical energy in the form of ATP in aerobic cells.

OXPHOS complexes are a mosaic from two genetic systems

Each of the OXPHOS complexes consists of several protein subunits and prosthetic groups. Most protein subunits, like the other roughly 1000 mitochondrial proteins [3], are encoded in the nucleus, synthesized in the cytosol
and post-translationally imported into the organelle (Figure 1A) [4,5]. However, a subset of the OXPHOS subunits is encoded in an organelle-resident mitochondrial genome [6,7]. The organelle contains a full genetic system to replicate, transcribe and translate the genes encoded in this genome.

Figure 1. (A) Mitochondria contain different membrane surrounded compartments. The inner mitochondrial membrane houses the OXPHOS system. The matrix contains an organelle-resident genome and a full genetic system for transcription and mRNA translation. Mitochondrial proteins are either imported from the cytosol through TOM (translocase of the outer membrane) and TIM (translocase of the inner membrane) complexes or synthesized inside the matrix (adapted from [8]). (B) The mitochondrial OXPHOS system. Electrons released from NADH and succinate oxidation in complex I (CI) and complex II (CII) are transferred via complex III (CIII) to complex IV (CIV). Here the electrons are used to reduce molecular oxygen to water. The electron transfer is coupled to an endergonic proton transfer in CI, CIII and CIV from the matrix to the intermembrane space (IMS). The energy of the generated proton gradient is used by complex (CV) to generate ATP.

How did the organelle acquire its own genome? According to the now widely accepted endosymbiont hypothesis mitochondria evolved from an α-proteobacterium that was incorporated into an α-archaical cell [9,10]. This endosymbiotic event was a key step in the evolution of aerobic eukaryotes [11]. During the course of evolution most of genes of the α-proteo-
bacterium were lost, due to a lack of evolutionary pressure or transferred to the nucleus, leaving behind the genes which constitute the current mitochondrial genome [12]. Interestingly, the genetic code was slightly altered after the endosymbiotic event [13]. In *Saccharomyces cerevisiae* (*S. cerevisiae*) six codons were reassigned and differ from the universal genetic code. In the human mitochondrial genome four codons are also used differently. Codon reassignments together with high hydrophobicity of mitochondrially encoded proteins were suggested as reasons why some genes resisted transfer to the nucleus [14].

The mitochondrial genome is organized and condensed within the matrix in a so called nucleoid [15,16]. The specific mitochondrial genes vary between organisms, but generally mitochondrial DNA encodes membrane protein subunits of the OXPHOS system, rRNA of the mitochondria specific ribosomes (mitoribosomes) and tRNAs necessary for translation. Therefore, the mitochondrial genome is dedicated towards the production of OXPHOS subunits (Figure 2A). The genome size can vary from 15 to 75 kb [17]. The size is not directly correlated to the number of genes that are encoded in the respective genome. For example, the 75 kb *S. cerevisiae* mitochondrial genome encodes 8 polypeptides [18]. All are membrane protein subunits of the OXPHOS system, with the exception of Var1, which is part of the small subunit of the mitoribosome (Figure 2B). On the other hand, the human mitochondrial genome is only 16.5 kb long and encodes 13 polypeptides, all of which are OXPHOS subunits [19]. The size of the mitochondrial genome is therefore dependent on the presence and constitution of non-coding regions. Long introns in the yeast mitochondrial genome account for the larger size, whereas the human mitochondrial DNA is condensed and contains only a small portion of non-coding sequences.
The expression of mitochondrial genes is not self-sufficient and requires nuclear gene products. Components necessary for transcription, RNA processing and translation are synthesized in the cytosol and post-translationally imported into the organelle (Figure 1A). Roughly 250 nuclear genes are required for mitochondrial gene expression [21]. How the OXPHOS subunits encoded in the mitochondrial genome are expressed, inserted into the membrane and assembled into functional complexes, will be discussed in the following chapters.
The mitoribosome

According to the endosymbiont hypothesis the mitoribosome evolved from an α-bacterial ancestor [9,10]. The bacterial ancestry of the mitoribosome is reflected in a few similarities between the bacterial and mitochondrial ribosomes. The overall organization into a large and small subunit, consisting of enzymatic rRNA and protein subunits, is similar between both systems. The rRNA and protein domains involved in decoding and peptidyl transferase catalysis show a high degree of conservation [20]. Additionally, both ribosomes are sensitive against the same groups of antibiotics [22,23].

Nevertheless, the mitoribosome has been extensively remodeled in the course of evolution. The rRNA sequences of bacterial and mitoribosomes differ significantly in length. The *S. cerevisiae* rRNA increased in size, by acquiring mitochondria specific expansion segments (ES). In the mammalian system the rRNA portion is dramatically reduced [24], leading to smaller rRNAs with lower sedimentation coefficients [25]. The rRNA evolution was accompanied in yeast and mammals by recruitment of additional mitochondria-specific ribosomal proteins. The protein content of the mitoribosome is further increased by N- and C-terminal extensions of the conserved protein subunits [26]. The addition of new protein subunits and extension of conserved ribosomal proteins, accompanied with the loss of rRNA segments in mammals, lead to a shift in the rRNA to protein ratio in mitoribosomes compared to the bacterial ribosome (Figure 3A).

The identity and exact number of mitoribosomal proteins was long under debate [27–30]. Due to the low abundance of mitoribosomes crystal structures could not be obtained, therefore mitoribosome subunits were identified by mass spectrometry [31–36] or inferred from homology [37]. Only the recent advancement in high resolution cryo-electron microscopy (EM) [38,39] allowed the determination of molecular models for the yeast and mammalian mitoribosomes [40–46]. In the structures the identities and positions of all mitoribosomal proteins were determined.
Figure 3. (A) Protein and RNA content of the bacterial ribosome, yeast and human mitoribosome (adapted from [25]). (B) Comparison of the central protuberance (red) of the bacterial ribosome with the yeast and human mitoribosomes (PDB 5DOX, 5MRC and 3J9M).

The mitoribosome structures revealed an architecture that differs substantially from the bacterial ribosome (Figure 3B). The 11 rRNA expansion segments in the large subunit of the yeast mitoribosome act as scaffold for the mitochondria-specific proteins and spread over the whole secondary structure [45]. These new elements occupy unique positions in the mitoribosome, restructuring for example intersubunit bridges [40]. In mammals the rRNA loss is not completely compensated by the newly recruited ribosomal proteins, giving the mammalian mitoribosome a more porous structure when compared to its bacterial counterpart [20]. The different protein and rRNA contents of the mammalian and yeast mitoribosomes become
also apparent in their structures. The mitoribosomal proteins occupy different positions, giving the different mitoribosomes distinct shapes (Figure 3B).

Mitochondria lack the 5S rRNA, which is part of the bacterial central protuberance in the large ribosomal subunit. In *S. cerevisiae* mitochondria the central protuberance is restructured by expansion segments and newly recruited mitoribosomal proteins. These new elements occupy the same position as the bacterial 5S rRNA, but give the central protuberance a different shape (Figure 3B)[45]. In mammals the missing 5S rRNA is compensated by recruiting a mitochondrial tRNA into the large ribosomal subunit [43,44]. Although the central protuberance was remodeled its functions in translation fidelity and inter-subunit communication seem to be conserved [44].

The small mitoribosomal subunit was also restructured after the endosymbiotic event. Because mitochondrial transcripts lack Shine-Dalgarno sequences, the 3’-end of the 15S and 12S rRNAs occupy different positions in the yeast and mammalian mitoribosomes [40–42].

After decades of studying the components and architecture of the mitoribosome, only in recent years the determination of high resolution cryo-EM structures solved some long standing questions in the field. These structures revealed surprising differences between the mitochondrial and bacterial translation machineries. Furthermore, species specific adaptions altered the structures of mitoribosomes from mammals and yeast. For this reason, it was proposed that the mitoribosomes co-evolved with the OXPHOS genes encoded in the mitochondrial genome [47]. Accordingly, the evolution happened in two phases, the rRNA expansion, witnessed in the yeast mitoribosome, preceded the reduction of the rRNA content in the current mammalian mitoribosome. During the rRNA reduction the recruitment of additional proteins continued, accounting for the higher protein content of the mammalian mitoribosome. However, the structures of the mitoribosomes are just the starting point for detailed molecular studies of translation in mitochondria. These will help in the future understanding processes like the
evolution of different mitoribosomes, mechanisms of membrane protein insertion and translation initiation or antibiotic side effects.

**Translational activators**

The concept of translational activators was proposed by Fox and Costanzo in 1990 [48]. Accordingly, in *S. cerevisiae* the translation of the eight mitochondrially encoded mRNAs is dependent on a set of transcript specific translational activators. These factors organize and regulate the translation for their respective client mRNAs. Translational activators are characterized by a functional [49] or physical interaction [50] with the 5´-untranslated region (UTR) of their client transcript and defective mitochondrial translation in their absence. Mature mitochondrial mRNAs are different from their cytosolic or bacterial counterparts. In mitochondria of *S. cerevisiae* the mature mRNAs do not contain a CAP structure or a 3´-end poly A tail. Instead the 5´- and 3´-ends of all mature transcripts contain long (54-954 bp) UTRs, that are required for their translation [51–57].

The 5´-UTR regulates the translation of the downstream coding sequence by a functional interaction with a specific translational activator. The absence of this translational activator can be compensated by exchanging the 5´-UTR with the 5´-UTR of another mRNA. In this case the authentic translational activator is replaced by the translational activator of the other mRNA [58]. Many translational activators were identified by fusing 5´-UTRs to reporter genes through biolistic transformation and screening for required nuclear gene products [59]. The exact molecular function and role in mitochondrial translation of the found translational activators have been studied in some cases. The following table summarizes the known translation activators and their proposed functions:
Table 1. Functions of *S. cerevisiae* translational activators (transl. act.).

<table>
<thead>
<tr>
<th>Client</th>
<th>Transl. act.</th>
<th>Function(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX1</td>
<td>Mss51</td>
<td>Peripheral membrane protein [60] that functionally and physically interacts with the COX1 mRNA 5'-UTR and coding region [61]. Regulates Cox1 synthesis through feedback loop [60].</td>
</tr>
<tr>
<td></td>
<td>Pet309</td>
<td>Integral membrane protein [62] that functionally [49] and physically [50] interacts with the COX1 mRNA 5'-UTR. Contains PPR domains, that are necessary for COX1 translation [63].</td>
</tr>
<tr>
<td></td>
<td>Mam33</td>
<td>Functional interaction with the COX1 mRNA 5'- or 3'-UTR, that is necessary for Cox1 synthesis [64].</td>
</tr>
<tr>
<td></td>
<td>Mss116</td>
<td>Interacts with Pet309 and is required for Cox1 synthesis. Also is involved in transcription and splicing [65].</td>
</tr>
<tr>
<td>COX2</td>
<td>Pet111</td>
<td>Firmly bound to the membrane [66]. Functionally interacts with predicted stem-loop structure [67] in the 5'-UTR of the COX2 mRNA [68,69]. Contains PPR domains [70] and is rate limiting for Cox2 synthesis [66]. Overexpression inhibits COX1 translation, probably by interacting with the translational activators of COX1 [71].</td>
</tr>
<tr>
<td>COX3</td>
<td>Pet154</td>
<td>Peripheral membrane protein [72], that functionally interacts with the COX3 mRNA 5'-UTR [73]. Physically interacts with Pet122 and Pet494 [74].</td>
</tr>
<tr>
<td></td>
<td>Pet122</td>
<td>Integral inner membrane protein [72], that functionally interacts with the COX3 mRNA 5'-UTR [73]. Functionally interacts with mS26 (Pet123) of the SSU [75].</td>
</tr>
<tr>
<td></td>
<td>Pet494</td>
<td>Integral inner membrane protein [72], that functionally interacts with the COX3 mRNA 5'-UTR [76] and is rate limiting for Cox3 synthesis [77].</td>
</tr>
<tr>
<td>COB</td>
<td>Cbp1</td>
<td>PPR protein [70], that stabilizes the COB mRNA by a functional interaction with the 5'-UTR [78,79]. Suggested to be necessary for COB mRNA translation in the absence of Pet127 [80].</td>
</tr>
<tr>
<td></td>
<td>Cbs1</td>
<td>Integral inner membrane protein [81] that functionally interacts with the 5'-UTR of the COB mRNA [82,83]. Interacts with actively translating ribosomes [84].</td>
</tr>
<tr>
<td></td>
<td>Cbs2</td>
<td>Peripheral membrane protein [81] that functionally interacts with the 5'-UTR of the COB mRNA [82]. Binds to mitoribosomes [85].</td>
</tr>
<tr>
<td></td>
<td>Cbp3/Cbp6</td>
<td>Bind ribosomes at tunnel exit and functionally interact with the 5'-UTR of COB mRNA [86]. Bind newly synthesized cytochrome b for feedback regulation [87].</td>
</tr>
<tr>
<td>ATP8/6</td>
<td>Aep3</td>
<td>Peripheral membrane protein that stabilizes the ATP8/6 mRNA [88].</td>
</tr>
<tr>
<td></td>
<td>Snt1</td>
<td>Represses translation of the ATP8/6 mRNA [89].</td>
</tr>
<tr>
<td>ATP6</td>
<td>Atp22</td>
<td>Functional interaction with the 5'-UTR of the ATP6 mRNA [90].</td>
</tr>
<tr>
<td>ATP9</td>
<td>Aep3</td>
<td>PPR protein that is required for ATP8 expression [91]. Required for mIF2-dependent initiation of mitochondrial mRNAs with unformylated initiator tRNAs [92].</td>
</tr>
<tr>
<td>ATP9</td>
<td>Aep1</td>
<td>PPR proteins [70], that functionally interact with the ATP9 mRNA and are required for Atp9 expression [93,94].</td>
</tr>
<tr>
<td></td>
<td>Aep2</td>
<td></td>
</tr>
<tr>
<td>VAR1</td>
<td>Sov1</td>
<td>PPR protein [70], that functionally interacts with the 5'-UTR of the VAR1 mRNA [95]. Belongs to group of mitochondrial translation control proteins that extend life span upon deletion [96].</td>
</tr>
</tbody>
</table>
Post-transcriptional regulation by translational activators

Apart from the interaction with the 5´-UTR, the functions of translational activators are diverse (Table 1). Therefore, different roles in post-transcriptional regulation by translational activators have been found and proposed:

(A) **mRNA-mitoribosome interaction:** As many translational activators directly interact with the ribosome [85] and in some cases the mRNA [50] it was proposed that they aid the mRNA-mitoribosome interaction, specifically for start site selection during initiation. Mature mitochondrial mRNAs lack Shine-Dalgarno sequences, which are important for translation initiation in bacteria [97]. Translational activators can compensate this loss by playing an active role in translation initiation. This hypothesis was supported by the presence of an unidentified electron density in the structure of the yeast mitoribosome [40]. The density contacts the mRNA exit canyon, a part of the ribosome that has been shown to functionally interact with 5´-UTRs [98]. Therefore, the authors proposed that the density represents a mixture of co-purified translational activators and the mRNA exit canyon acts as platform for the binding of translational activators to the yeast mitoribosome.

(B) **Localization of the mRNA to the inner mitochondrial membrane:** Almost all translational activators are integral membrane proteins or peripherally associated with the inner mitochondrial membrane (Table 1). Therefore, translational activators could recruit mRNAs to the membrane by binding the 5´-UTR of their client transcript. However, it is not clear at the moment if all translational activators physically interact with their client mRNA.

(C) **Stabilization of mRNA:** Cbp1 is necessary for cytochrome b (COB) mRNA stabilization [78,79]. Many other translational activators are required for accumulation of their client transcript. Pet309 is necessary for accumulation of the COX1 mRNA [49]. However, Pet309 is only required in the presence of introns in the COX1 mRNA. Since the
splicing of mitochondrial transcripts depends on translation by the mi-
toribosome, it is not clear if this destabilization originates from the ab-
sence of the translational activator or a splicing defect. The intronless
\textit{COX2} transcript is destabilized in the absence of Pet111 [68]. But, in
this case it is not clear if the translational activator directly stabilizes the
mRNA or the translation inhibition generally destabilizes mitochondrial
transcripts. It remains to be elucidated, if some translational activators
directly stabilize their client mRNA, like the Cbp1 protein.

(D) \textbf{Coordination of nuclear and mitochondrial gene expression:} The trans-
lational activator Mss51 binds to the newly produced Cox1 after syn-
thesis [61]. Mss51 stays bound to its client protein until a certain inter-
mediate in the assembly of complex IV is formed. The formation of
the assembly intermediate depends on the import of nuclear imported
subunits. Until the intermediate is assembled the translational activator
is sequestered in the assembly process and not free to induce new
rounds of Cox1 synthesis [60]. This type of regulation represents an el-
egant way of coordinating nuclear and mitochondrial gene expression
during the assembly of OXPHOS complexes and will be discussed in
detail on the example of cytochrome \textit{b} biogenesis.

(E) \textbf{Rate limit for mitochondrial translation:} Some translational activators
are rate limiting for the translation of their client mRNA [77]. There-
fore, the availability of a translational activator represents an important
part of post-transcriptional gene regulation in mitochondria. Further-
more, it was shown that yeast mitochondrial transcript levels are not
changing dramatically in response to a shift to a non-fermentable car-
bon source [99]. Accordingly, mitochondrial post-transcriptional gene
regulation and translational activators seem to be more important for
respiratory growth than transcriptional activation of OXPHOS subu-
nits.

(F) \textbf{Organization of respiratory chain assembly:} The translational activators
of the \textit{COX1}, 2 and 3 mRNAs have been shown to interact physically
on the inner mitochondrial membrane [100]. On this basis, it was hypothesized that they organize complex IV assembly prior synthesis of mitochondrially encoded complex IV subunits. Through the physical interaction of the COX mRNA translational activators, the synthesis of complex IV subunits is guided to the same compartment. Interestingly, the COB and COX1 mRNA translational activators Cbp1 and Pet309 were found together in a large complex [101]. As complex III and IV build higher order assemblies in the form of supercomplexes, this interaction could direct the translation of both complexes of the same compartment for efficient complex formation [102]. Furthermore, Pet309 interacts directly with the mitochondrial transcription and maturation machinery, suggesting a direct link of transcription, maturation and translation of mitochondrial mRNAs [100,101].

**Cytochrome b biogenesis**

Cytochrome b synthesis illustrates several features by which translation activators regulate mitochondrial protein synthesis and will be therefore discussed in detail. The translation of the COB mRNA depends on five transcript specific translational activators. Cbp1 was shown to stabilize the mature COB mRNA by interacting with the 5'-UTR, prior to translation [78,79]. The translational activators Cbs1 and Cbs2 are integral and peripheral membrane proteins, respectively [81]. Both proteins functionally interact with the COB 5'-UTR [82,83] and the mitoribosome [84,85]. Cbs1 and Cbs2 probably recruit the COB mRNA to the ribosome and the inner mitochondrial membrane.

The translational activators Cbp3 and Cbp6 build a complex that interacts with the COB 5'-UTR and the mitoribosome [86]. The Cbp3/6 complex interacts with the newly produced cytochrome b after synthesis [87]. Upon hemylation and Cbp4 interaction with cytochrome b, Cbp3/6 dissociates from the complex III assembly intermediate [103]. If assembly of cytochrome b is arrested the Cbp3/6 complex is sequestered in the assembly intermediate and consequently cytochrome b synthesis is downregulated
Therefore, the Cbp3/6 complex is not only required for translation of the COB mRNA, but also for regulating cytochrome b synthesis according to the efficiency of complex III assembly. Since the assembly of complex III depends on nuclear encoded subunits and assembly factors, this feedback loop couples mitochondrial to nuclear gene expression.

Figure 4. Cytochrome b synthesis is regulated by the Cbp1,3,6 and Cbs1,2 translational activators. After protein synthesis Cbp3/6 binds to cytochrome b until hemylation is completed and Cbp4 binds to cytochrome b. As long as Cbp3/6 is sequestered in the assembly process COB mRNA translation is downregulated.

Translation activators in mammals
Mammalian mitochondrial transcripts differ from the described yeast mRNAs. They lack long 5´-UTRs and contain 3´-end poly-A tails [104,105]. As the interaction with the 5´-UTR is one of the defining characteristics of translational activators, it is not surprising that only a few homologs have been found in the mammalian system. The LRPPRC (Leucine-rich PPR motif-containing) protein is a distant homolog of Pet309 and mutations cause French-Leigh syndrome, which is characterized by complex IV deficiency [106]. It was shown that the LRPPRC protein is involved in post-transcriptional gene regulation in mammalian mitochondria by influencing mRNA stability, polyadenylation and translation [107,108]. Contrary to the yeast translation activators, the LRPPRC protein is not client specific. The TACO1 (Translational activator of Cox1) protein was proposed to be a human translation activator of Cox1 [109]. No yeast homolog of TACO1
was found so far, but unlike the LRPPRC protein it seems to be specific for the synthesis of Cox1. How TACO1 modulates Cox1 synthesis is currently not well understood. The human homologs of Cbp3 (UQCC1) and Cbp6 (UQCC2) seem to play a similar role as their yeast counterparts. UQCC1 and UQCC2 form a stable complex that interacts with newly synthesized cytochrome \( b \) [110]. Additionally, in the absence of UQCC2 cytochrome \( b \) synthesis or stability is decreased.

The different evolution of yeast and mammalian mitochondria is reflected in different genomes, transcripts and ribosome structures. Therefore, although some proteins with similarities to yeast translational activators have been described, it is likely that mitochondrial translation is regulated differently in both systems.

**Mitochondrial membrane protein insertion**

**Mitoribosome membrane attachment**

In bacteria ribosomes are targeted to the membrane for protein insertion by a dedicated process [111]. Nascent chains destined for co-translational membrane insertion contain N-terminal signal sequences. These sequences are recognized by the signal recognition particle (SRP) which targets the ribosome nascent chain complex to the membrane by interacting with the SRP receptor. Homologs for all these key components are absent in mitochondria [112]. Additionally, mitoribosomes seem to be permanently attached to the inner mitochondrial membrane [113–116] and contrary to bacteria, the interaction with the membrane is independent of the nascent chain [117]. Since mitochondrial ribosomes produce almost exclusively membrane proteins, this adaptation of the ribosome membrane interaction probably occurred to accommodate the membrane insertion of mitochondrially encoded proteins [118].
How is the mitoribosome attached to the inner mitochondrial membrane in the absence of the SRP and SRP receptor? The high resolution structure of the yeast large ribosomal subunit revealed a membrane facing protuberance, consisting of an mitochondria specific rRNA expansion segment (Figure 5A)[45]. Cryo-electron tomography of yeast mitochondria showed that this expansion segment (96-ES1) is one of two contact sites of the mitoribosome.
some with the inner mitochondrial membrane [116]. The second contact site is not part of the mitoribosome, but the peripheral membrane protein Mba1 (Figure 5B). Biochemical evidence showed that Mba1 acts as a mitoribosome membrane receptor [113, 114]. On this basis, it was proposed that the interaction with Mba1 tethers the mitoribosome to the inner mitochondrial membrane and aligns the tunnel exit for insertion into the membrane. The mL45 protein is the human homolog of Mba1. It is a permanent ribosomal subunit and occupies a similar site on the mitoribosome (Figure 5A) [46]. The mammalian mitoribosome lacks the yeast rRNA expansion segment (96-ES1). Tomography studies of human mitochondria confirmed that the mL45 protein is the only membrane contact site of the human mitoribosome [119]. As the overall orientation of the large ribosomal subunit was similar in yeast and human, the Mba1/Mrpl45 proteins seem to play a general role in the mitoribosome membrane attachment. However, the second contact point of the yeast mitoribosome and the dynamic mitoribosome association of Mba1 indicate different adaptations in both systems. Additionally, it seems that the position of the tunnel exit was altered in the yeast mitoribosome (Figure 5A). Accordingly, the path of the tunnel is different in the LSU of the yeast mitoribosome.

**Mitochondrial membrane protein insertion**

Mitochondria not only lack the membrane targeting machinery, but also the protein conducting Sec channel [112]. Instead, insertion of *S. cerevisiae* mito-chondrially encoded proteins into the inner mitochondrial is carried out by Oxa1 [120]. This protein is universally conserved and belongs to the YidC/Oxa1/Alb3 family, with members in bacteria, mitochondria and chloroplasts [121]. All members are characterized by five transmembrane segments responsible for membrane protein insertion [122]. The soluble C-terminal α-helical domain of Oxa1 binds to the mitoribosome and is required for membrane protein insertion [123]. Crosslinking studies mapped the interaction site close to the tunnel exit proteins uL23 and uL24 [26, 124, 125]. This biochemical data is in line with a cryo-EM structure of Oxa1 reconstituted with the *Escherichia coli* ribosome, in which Oxa1 binds...
to the bacterial homologs of uL23 and uL24 [126]. Furthermore, Oxa1 interacts directly with nascent polypeptide chains emerging from the tunnel exit, probably to support co-translational membrane protein insertion [113]. The previously discussed ribosome receptor Mba1 is also a component of the mitochondrial membrane protein insertion machinery. Mba1 interacts directly with nascent chains and is required for efficient membrane insertion of Cox2 [113]. Genetic evidence also points to a role of $MBA1$ in membrane insertion, as $MBA1$ functionally interacts with the C-terminal domain of Oxa1[114]. Mba1 forms a complex with Mdm38 [127], another inner membrane protein that binds to the yeast mitoribosome and Oxa1 (Figure 5C) [128]. The molecular mechanism by which Oxa1 and Mba1 insert mitochondrially encoded proteins into the inner membrane is currently not fully understood, but will be discussed in the following chapter on the example of Cox2.

**Cox2 membrane insertion**

A stem loop structure in the 5’-UTR of the *S. cerevisiae* COX2 mRNA is recognized by Pet111 [68,69]. This translational activator is membrane-bound and recruits the COX2 mRNA to the inner mitochondrial membrane [67–69]. The newly synthesized Cox2 protein (pCox2) consists of an N-terminal signal sequence, two transmembrane domains and a C-terminal tail. After successful translocation across the inner mitochondrial membrane the N-terminal sequence is cleaved off by the inner membrane peptidase complex, consisting of Imp1/2 and Som1 [129–131]. The processed Cox2 (mCox2) is then assembled into complex IV. During and after synthesis Oxa1 is required for efficient export of the soluble N- and C-terminal tails across the membrane to the intermembrane space [132]. The group of Thomas Fox established by an elegant reporter system that the Oxa1 dependent export of the N-terminus is independent of membrane potential, but the C-terminus requires it, suggesting that both domains are exported by a distinct mechanism [133].
Figure 6. Cox2 translation is regulated by the translational activator Pet111. The Oxa1 and Mba1 proteins are necessary for the export of the N-tail across the membrane. After successful translocation the N-terminal signal sequence is delivered to the Imp1/Imp2/Som1 complex by Mba1 and Cox20. The C-tail is translocated subsequently across the membrane. This process is dependent on Cox18, Mss2, Pnt1, Cox20 and Oxa1. Cox2 biogenesis is completed by the formation of Cu₄ center.

While Oxa1 is required for N-and C-terminal translocation of Cox2, the Cox18 protein is specifically necessary for the export of the C-tail [134]. Cox18 is an integral membrane protein with a role in Cox2 accumulation and complex IV assembly [135]. It was shown that Cox18 interacts with Mss2 and Pnt1 [134]. Both proteins are necessary for the export of the Cox2 C-tail [136,137]. A role of the Cox18/Mss2/Pnt1 complex in protein translocation is supported by the fact that Cox18 is a member of the YidC/Alb3/Oxa1 family [138]. Interestingly, Cox18 lacks the C-terminal ribosomal binding site of Oxa1. Translocation of the C-tail was suggested to happen post-translationally and in this case the interaction with the mitoribosome would not be required [138].

Efficient translocation of Cox2 across the inner mitochondrial membrane also depends on Mba1. Upon deletion of MBA1 the pCox2 form accumulates and cells grow slower on respiratory media [114,127]. As discussed before, Mba1 interacts directly with the large ribosomal subunit aligning the tunnel exit for membrane protein insertion [116].
The Cox20 protein is another factor that is required for Cox2 biogenesis [139]. This membrane-bound chaperone binds to the newly synthesized pCox2 and is required for N-terminal processing as well as C-tail export [140]. Furthermore, Cox20 interacts with the mitoribosome, Mba1 and Cox18 in a Cox2 dependent manner [141]. Therefore, it was suggested that Mba1 together with Cox20 plays an active role in delivering pCox2 to the C-tail export machinery (Cox18/Mss2/Pnt1) post-translationally (Figure 6). Afterwards Mba1 dissociates from pCox2 and the inner membrane peptidase (Imp1/2, Som1) complex cleaves the N-terminal signal sequence. The Cox18/Mss2/Pnt1 complex together with Cox20 exports the C-tail into the intermembrane space and biogenesis of Cox2 is completed by formation of the Cu$_A$ center by the metallochaperone Sco1 [142].

Degradation of mitochondrially encoded membrane proteins

Mitochondrial quality control

The mitochondrial quality control system is responsible for protein biogenesis and homeostasis in the organelle. The degradation of mitochondrially encoded proteins is carried out by a set of organelle-specific chaperones and proteases [143]. The proteases of the mitochondrial quality control system belong to the AAA+ (ATPases associated with various cellular activities) superfamily of proteins [144]. Members are characterized by a 200-250 amino acids long ATP-binding (AAA) domain consisting of a Walker A and B motif and other conserved domains [145]. The AAA domain provides the energy for conformational changes (unfolding and membrane extraction) of substrate proteins, which are degraded by a proteolytic domain. Mitochondrial AAA proteases are classified into three families, according to their bacterial homologs:

(A) The Lon/Pim1 proteases degrade oxidatively damaged proteins in the mitochondrial matrix [146,147].

(B) The mtClpXP protease is only present in mammals with no homologs in yeast mitochondria. The exact function of the protease complex is currently not understood [148].
Mitochondrial FtsH homologs are membrane-bound proteases, which are divided into m-AAA and i-AAA proteases, dependent on the localization of their active site in the matrix or intermembrane space [149].

Membrane-bound mitochondrial AAA proteases
In yeast mitochondria non-assembled inner membrane proteins are degraded by the hetero-oligomeric m-AAA protease complex, that consists of Afg3 (or Yta10) and Yta12 (or Rca1) complex [150]. Degradation of mitochondrially encoded membrane proteins by the m-AAA protease is negatively regulated by the Phb1 and Phb2 proteins [151]. Both proteins form a complex that has been suggested to function as membrane-bound chaperone of newly synthesized mitochondrial translation products, protecting them for premature degradation [152]. On the other hand, the Phb1/Phb2 complex was suggested to act as membrane scaffold, defining functional subcompartments at the inner mitochondrial membrane [153]. How this function alters the degradation of proteins at the inner mitochondrial membrane by the m-AAA protease is currently not understood.

The i-AAA protease is a homo-oligomeric complex, consisting of the Yme1 membrane protein [154,155]. Yme1 is positively regulated by the Mgr1 and Mgr3 complex [156,157]. The complex binds directly to the i-AAA protease and probably aids substrate binding. Since the function of Mgr1 and Mgr3 was only studied on model substrates, it was not known which proteins in the inner mitochondrial membrane depend for their proteolysis on these regulators of the i-AAA protease. However, a recent study found that degradation of mitochondrial outer membrane proteins depends on the Mgr1/Mgr3 complex and the i-AAA protease [158]. Substrates at the inner mitochondrial membrane are to this date not identified.
Since the catalytic domains of the AAA proteases are on opposite sides of the inner mitochondrial membrane, it was assumed that the substrates of the proteases are recognized either in the intermembrane space or the matrix. However, the group of Thomas Langer showed that the m-AAA and i-AAA protease were able to degrade the same model substrate [159]. Accordingly, substrate transmembrane segments need to be extracted by the proteases prior or during degradation [160]. The substrate recognition by both membrane-bound AAA proteases is degenerate and seems to be only dependent on the presence of unfolded soluble domains in the intermembrane space or the matrix [161]. The Pim1/Lon protease is a high copy number suppressor of the m-AAA protease, suggesting that in the absence of membrane-bound proteases the soluble AAA protease can degrade membrane-bound substrates [162]. If this is happening in vivo or how the different substrates are delivered to the proteases is currently not known.

Several substrates of the i-AAA protease have been determined so far. A genetic study found YME1 (the i-AAA protease) among other genes to be necessary for Cox2 degradation, when assembly of complex IV is inhibited [163]. Degradation of Cox2 by the i-AAA protease requires the previously
discussed chaperone Cox20 [164]. In the absence of cytochrome c, Cox2 and Cox3 cannot be assembled into complex IV and their degradation depends on the i-AAA protease [165]. Interestingly, Cox3 was also described as substrate for the m-AAA protease, together with Cox1 and cytochrome b [165]. Furthermore, the m-AAA protease is required for degradation of non-assembled Atp9 and carries out a role in the assembly of the ATP synthase (Figure 7) [150].

The m-AAA complex also controls respiratory chain formation [166]. Furthermore, processing of the intron containing COB and COX1 transcripts seems to be dependent on the presence of the m-AAA protease. It is currently not known how the m-AAA protease influences splicing of these mitochondrial transcripts. The dual role in degradation and assembly of respiratory chain subunits is interesting but also complicates the studies of mitochondrial membrane protein degradation. The absence of the m-AAA protease can be compensated by high copy numbers of the mitochondrial membrane insertion machinery, Oxa1 and Mba1 [162]. But, as both proteins are also involved in respiratory chain assembly it is not clear at the moment which function the high copy numbers are suppressing [141,167,168]. The bacterial homologs of the m-AAA protease, Oxa1 and the regulators Phb1/Phb2 are forming a complex [169]. The Oxa1 homolog YidC apparently acts as a chaperone aiding degradation of misassembled membrane proteins. Therefore, bacterial quality control appears to happen very early during or after membrane protein insertion. If the mitochondrial quality control system and membrane protein insertion machinery also interact physically is currently not known. However, the discussed genetic evidence points to a functional interaction. It remains an exciting task to study how mitochondrial protein synthesis, membrane insertion, respiratory chain assembly and quality control are connected and regulated.
Aims

Mitochondria are responsible for the expression of a handful of OXPHOS subunits. Transcription and translation of the genetic information in the mitochondrial genome is therefore critical for ATP production by OXPHOS. The mitoribosome was extensively remodeled in the course of evolution. All adaptations lead to a translation machinery dedicated towards production of a small amount of hydrophobic proteins in an oxidatively challenging environment. The organelle-specific translation regulation mechanisms are currently not well understood, as they are not strongly related to the bacterial system.

The studies presented in this thesis focused on the yeast mitoribosome and protein interaction partners. Despite many efforts and decades of research in mitochondrial translation, to this date no \textit{in vitro} translation system could be established. This causes a number of open questions concerning the exact molecular function of proteins that interact with the mitoribosome. Furthermore, the mitoribosome is less stable and abundant compared to bacterial counterparts, making biochemical studies of the mitochondrial translation apparatus challenging. Therefore, this thesis was aimed at establishing robust biochemical tools for the analysis of the yeast mitoribosome. These tools were designed in order to find and study the molecular functions of mitoribosome interaction partners and consequently understand the mitochondria specific adaptations of the translation machinery. Working with the facultative anaerobe \textit{S. cerevisiae} allowed me to study the function of components that are essential for respiratory growth.
Summary of papers

Paper I: Organization of mitochondrial gene expression in two distinct ribosome-containing assemblies

Mitochondria contain an organelle specific genome and house all components necessary for transcription, RNA maturation and translation of the encoded genetic information. In this study we established conditions that allowed the efficient isolation of intact mitoribosomes and interacting proteins.

Figure 8. Experimental procedure for the purification of the mitoribosome and interactors. Proteins were identified after immunoprecipitation (IP) by label free quantitative mass spectrometry and protein classes represented in a pie chart (LS, low salt; HS, high salt; LSU, large subunit; SSU, small subunit; CR, chaperones; DNA, proteins involved in DNA metabolism; ME, metabolic enzymes; OXPHOS, components of the oxidative phosphorylation system; PA, proteases; RNA, proteins involved in RNA metabolism; TL, translation; UC, uncharacterized proteins)
We found a vast and diverse interactome of the *S. cerevisiae* mitoribosome organized in large assemblies (Figure 8). These assemblies probably escaped detection so far, as the mitoribosome is very unstable during isolation and native interactions were lost in the process. We determined that factors involved in mRNA metabolism, mitoribosome assembly, DNA metabolism together with translational activators, chaperones and proteases are physically associated with the mitoribosome. Accordingly, we termed these assemblies MIOREX, for mitochondrial organization of gene expression. STED microscopy of yeast mitochondria visualized two different subsets of MIOREX complexes. One subset is very close to the mitochondrial nucleoid, organizing all steps of mitochondrial gene expression in close proximity. The exact role of the different MIOREX complexes is currently not known. They could serve as platforms in order to channel mRNAs from transcription to translation within one complex. The organization into higher order complexes is a common theme in mitochondria. Respiratory chain complexes, ER-mitochondria contact sites and protein import machineries are all organized in large assemblies. The exact necessity of these organelle specific adaptations remains to be elucidated but demonstrate the striking differences between the mitochondrial and bacterial evolution.

**Paper II: The ribosome receptors Mrx15 and Mba1 jointly organize cotranslational insertion and protein biogenesis in mitochondria**

The yeast mitoribosome almost exclusively produces membrane protein subunits of the OXPHOS complexes. This specialization allowed many mitochondria specific adaptations of the membrane protein insertion mechanism. First, the mitoribosome is permanently attached and not targeted to the inner mitochondrial membrane. The interaction with the membrane depends on the mitoribosome receptor Mba1. This protein interacts with the mitoribosome and is in direct contact with nascent polypeptide chains. Second, protein translocation in mitochondria is independent of the Sec translocon. Instead proteins are inserted into the membrane by Oxa1.
In this study we refined the biochemical analysis of mitoribosome interactors, initiated in paper I (Figure 9A). This allowed us to establish subunit-specific interactomes of the large and small ribosomal subunits (Figure 9B). Among the interactors of the large subunit we found a previously uncharacterized protein, that we renamed Mrx15. We showed that Mrx15 is a membrane protein in the inner mitochondrial membrane and interacts with the ribosome via a soluble C-terminal domain. Furthermore, chemical cross-linking established that Mrx15 contacts nascent polypeptides chains. The MRX15 gene is dispensable for respiratory growth, but required upon simultaneous deletion with MBA1. In the absence of Mrx15 and Mba1 the mitoribosome interacts less efficiently with the inner mitochondrial membrane and the precursor form of Cox2 accumulates. This leads to a global complex IV deficiency causing the respiratory deficient phenotype. On this basis, we proposed an overlapping role of Mba1 and Mrx15 in mitoribosome membrane attachment and co-translational membrane protein insertion. This study also allowed us to assign the interacting subunits of known interactors of the ribosome. As the function of most ribosomal interactors is poorly understood, this will help future studies in establishing the exact molecular function of these proteins.
Paper III: Insertion defects of mitochondrially encoded proteins burden the mitochondrial quality control system

Mitochondrially encoded proteins are degraded by AAA proteases. These proteases are part of the mitochondrial quality control system, which is responsible for proteostasis and degradation of non-native polypeptides. Membrane-bound AAA proteases are large oligomeric complexes and characterized by the position of their active site in the intermembrane space (i-AAA) or matrix (m-AAA). The activity of either AAA-protease is modulated by a class of protease specific regulators. In this study, we combined yeast genetics with disk diffusion assays and in organello translation to study the mitoribosome receptors Mba1 and Mrx15. We found that in the absence of Mrx15, yeast cells become more resistant against proteotoxic stress. Mba1 was shown to have overlapping functions with Mrx15, but yeast cells become hypersensitive against proteotoxic stress in the absence of Mba1. This result implied different functions of Mba1 and Mrx15, apart from their role in membrane protein insertion. To further characterize the role of the ribosome receptors in stress tolerance we tested functional interactions of MRX15 and MBA1 with components of the mitochondrial quality control system. We found a mild growth retardation on respiratory media upon simultaneous absence of Mrx15 and either of the m-AAA protease regulators Mgr1 and Mgr3. However, analogous to cells lacking Mba1, yeast cells became hypersensitive against proteotoxic stress upon deletion of MGR1 or MGR3. On the other hand, MBA1 showed functional interactions with the regulators of the i-AAA and m-AAA proteases, MGR1, MGR3 and PHB1. The functional connection between the mitochondrial membrane protein insertion and quality control machineries suggests an early regulatory mechanism, directing mitochondrially encoded proteins during or shortly after synthesis towards OXPHOS complex assembly or degradation.
Paper IV: Organization of cytochrome b synthesis in yeast mitochondria

In paper I we identified the vast and diverse protein interactome of the yeast mitoribosome in the MIOREX complex. Among the interactors we found almost all known translational activators. We therefore investigated subsequently how the translational activator-mitoribosome interaction is modulated by the presence or absence of their client mRNA. For this purpose we split the ribosomal subunits by magnesium depletion and separated them subsequently on a sucrose gradient. We checked by northern and western blotting, if mRNAs and translational activators co-migrated with the small or the large subunit (Figure 10).

Surprisingly, first we discovered that not all mitochondrial mRNAs are interacting with the small ribosomal subunit. As translational initiation usually begins by loading the mRNA on the small ribosomal subunit we expected...
all mitochondrial transcripts to be associated with the small subunit. However, we demonstrated that the COB mRNA co-sediments with the large subunit in a sucrose gradient. On the other hand the ATP9 mRNA co-
sedimented with the small ribosomal subunit. Next we tested how the transl-
lational activators of the COB and ATP9 mRNAs interact with the mitori-
boosome. We showed that all known cytochrome b translational activators
interacted with the large subunit, the ATP9 mRNA translational activators
with the small subunit. To test if the interaction with the large subunit is
mediated by the mRNA or the translational activators we studied the trans-
lational activator-mitoribosome interaction in the absence of the COB
mRNA. All translational activator remained bound to the large subunit, with
the exception of Cbs1. In order to gain an understanding how Cbs1 is loca-
ized on the mitoribosome we employed a proximity based labeling ap-
proach. The experiment revealed that Cbs1 is closely localized to proteins
of the small subunit and the tunnel exit. All these results implied a dynamic
interaction of Cbs1 with the mitoribosome. On this basis, we suggested a
subunit-specific interaction of translational activators with either the small
or large subunit of the mitoribosome. This interaction is, in the case of cy-
tochrome b synthesis, independent of the mRNA and therefore pre-
organizes the translation of one specific transcript, prior loading onto the
small ribosomal subunit.
Conclusions and outlook

Mitochondria contain organelle-resident ribosomes, which are responsible for the translation of mitochondrially encoded mRNAs. The mitoribosome was adapted in the course of evolution to optimize the expression of mitochondrially encoded membrane proteins. The adaptations of the protein and rRNA content were accompanied by new protein interaction partners of the mitoribosome. The identity and function of the mitoribosomal interaction partners was the main subject of the thesis. The work presented here revealed an overall organization of gene expression in mitochondria in the MIOREX complex, new components responsible for membrane protein insertion and mitoribosome membrane attachment, a functional link of membrane protein insertion and protein degradation and the regulation mechanism of cytochrome b synthesis by translational activators.

Organization of mitochondrial gene expression by the MIOREX complex

The MIOREX complex represents a new conceptual framework of gene expression organization. Transcription and translation of nuclear encoded genes are spatially separated. After transcription the mRNA is exported out of the nucleus and translation is carried out in the cytosol. The MIOREX complex suggests that freely diffusing mRNAs do not exist in mitochondria. Instead, mRNAs are channeled after transcription to the mitoribosome. This hypothesis is supported by a study in which mRNAs were artificially introduced into mitochondria, failed to be translated [170]. We propose that these mRNA fail to be translated, because they are not channeled to the mitoribosome within the MIOREX complex.

Fluorescent microscopy studies in mammalian cells identified granules in mitochondria that are located closely to the mitochondrial genome. Because these granules are enriched in newly synthesized RNA and factors for RNA processing and decay, they were named mitochondrial RNA granules [171–
Components responsible for mRNA maturation and mitoribosome assembly are also part of the mitochondrial RNA granules [175–177]. A subset of ribosomal proteins was also found within the mitochondrial RNA granules. It was suggested that ribosome assembly occurs inside the granules. This is different from the MIOREX complex, where we find fully intact, assembled mitoribosomes. Due to the inherent instability of the mitoribosome it is not clear at the moment, if the entire mitoribosome is present in the granules or the found ribosomal subunits are only engaged in assembly. There is a striking functional overlap among the other components found in the MIOREX complex and mitochondrial RNA granules. Nevertheless, it is not known if the mitochondrial granules fulfill the same function as the MIOREX complexes in mitochondrial gene expression. Further studies employing the biochemical tools developed for the MIOREX isolation are necessary to determine if the mammalian mitochondria also employ a higher order organization of all steps of gene expression.

How MIOREX complexes are internally organized is completely unknown. Work presented in this thesis already started determining the role of some uncharacterized MIOREX components (paper II and III), determining the function of the other MIOREX parts remains an exciting task for the future.

**Functions of Mrx15 and Mba1 in protein biogenesis**

The MIOREX component Mrx15 was shown to interact with the large ribosomal subunit. Mrx15 is an integral membrane protein and interacts with the mitoribosome through a soluble C-terminal domain. Genetic evidence confirmed overlapping functions of Mrx15 with the ribosome receptor Mba1. We showed that in the absence of Mrx15 and Mba1 the mitoribosome interacts less efficiently with the inner mitochondrial membrane. Nevertheless, a significant portion of the mitoribosome is still attached to the membrane. The high resolution structure and cryo-electron tomography of the yeast mitoribosome showed that one point of membrane attachment
consists of a mitochondria-specific rRNA expansion segment [45,116]. Our results suggest that this segment is sufficient to attach a certain portion of the large ribosomal subunit to the inner mitochondrial membrane. This hypothesis was supported by a study showing that the large subunit is already membrane-bound during mitoribosome assembly [178]. Membrane attachment is only dependent on the incorporation of the 16S rRNA, where the previously mentioned expansion segment is located. This suggests a more specific role of Mba1 and Mrx15 in mitoribosome membrane attachment. This role is probably related to membrane protein insertion, as indicated by the interaction with nascent chain of both proteins.

The functions of Mba1 and Mrx15 are not completely redundant. For example Mrx15 cannot fully compensate the absence of Mba1. Furthermore, our experiments showed that Mrx15 is quantitatively attached to the large ribosomal subunit, whereas Mba1 dissociates from the mitoribosome even under mild salt conditions. This is in line with a recently discovered function of Mba1 in Cox2 maturation [141]. The authors showed that Mba1 escorts the newly synthesized Cox2 from the ribosome to the inner membrane peptidase for proteolytic cleavage. It is tempting to speculate that Mrx15 replaces Mba1 upon dissociation form the mitoribosome for efficient membrane protein insertion. However, it is currently not clear if both proteins are able to bind simultaneously to the mitoribosome.

The absence of Mba1 and Mrx15 mostly affected Cox2 biogenesis. We showed that the Cox2 processing is defective when both proteins are missing. Processing depends on the co-translational export of the Cox2 N-tail by Mba1 [114,141]. Therefore, Mrx15 is probably necessary for co-translational export of Cox2. However, a role of Mrx15 in post-translational export of the C-tail by the Cox18/Mss2/Pnt1 cannot be excluded at this stage [134,136–138]. Furthermore, if the functions of Mrx15 and Mba1 are limited to Cox2 biogenesis, or if both proteins play a more general role in mitochondrial membrane protein insertion is currently not known.
We tested the sensitivity of yeast cells against proteotoxic stress in the absence of either Mrx15 or Mba1. We found opposite effects on growth in their absence. Cells lacking Mba1 became hypersensitive against the induced stress, whereas cells lacking Mrx15 could grow at all tested concentrations of the employed antibiotic. This result confirmed independent functions of Mba1 and Mrx15. Interestingly, Mba1 was initially discovered as a multi-copy bypass of the m-AAA protease [162]. The m-AAA protease is a component of the mitochondrial quality control system and responsible for the degradation of non-native polypeptides [144]. Additionally we showed that \( \textit{MBA1} \) functionally interacts with regulators of the i-AAA and m-AAA proteases. All this data is consistent with a role of Mba1 in the turnover of mitochondrial encoded proteins.

Subsequently, we tested functional interactions between Mrx15 and regulators of the mitochondrial AAA proteases. In contrast to \( \textit{MBA1} \), we found only interactions with the regulators of the i-AAA protease, \( \textit{MGR1} \) and \( \textit{MGR3} \). Both Mgr1 and Mgr3 promote turnover of non-native mitochondrial proteins by the i-AAA protease [156,157]. As Mrx15 is responsible for Cox2 biogenesis, this functional interaction indicates turnover of Cox2 is dependent on Mgr1 and Mgr3. This is supported by several studies, in which Cox2 was shown to be degraded by the i-AAA protease [163–165].

How the functions of Mba1 and Mrx15 in protein turnover relate to their roles in membrane protein insertion is not understood. However, it is tempting to speculate that nascent chains are directed towards biogenesis or degradation during or shortly after synthesis. The mitochondrial quality control system could act during this early stage to avoid the accumulation and aggregation of hydrophobic proteins. In the MIOREX complex we found the m-AAA and i-AAA proteases together with the above mentioned regulatory proteins, suggesting that the mitochondrial quality control system is in close proximity to the mitoribosome. Additionally, the bacterial homologs of the m-AAA protease and Oxa1 interact [169], showing that quality control of bacterial inner membrane proteins occurs during translocation. Further studies will be necessary to test if mitochondrial quality control
system directly interacts with the mitoribosome in order to probe the integrity of nascent polypeptides.

**Control of cytochrome $b$ synthesis by translational activators**

In paper IV we found a subunit-specific interaction of translational activators and the mitoribosome. As this interaction is independent of the mRNA, we suggest that the synthesis of mitochondrially encoded protein is pre-organized on the mitoribosome by the respective translational activators. This would create a subset of specialized ribosomes that are dedicated towards the synthesis of one specific protein. This hypothesis was already proposed in 2010 and is supported by the data presented in paper IV [8].

As discussed previously, the role of the $COB$ mRNA translational activators Cbp3 and Cbp6 is understood very well. Both proteins form a complex that regulates cytochrome $b$ synthesis through a feedback loop [87]. But, how the Cbp3/6 complex initiates the translation of $COB$ mRNA is still not clear. Cbp3 was shown to bind to the tunnel exit of the large ribosomal subunit [86]. Initiation of translation is executed on the small subunit. It is therefore puzzling how the Cbp3/6 complex can induce translation of the $COB$ mRNA. By determining how the $COB$ mRNA and translational activators interact with the mitoribosome we discovered a subunit specific organization of cytochrome $b$ synthesis. Most importantly Cbp3 and Cbs1 were not able to bind simultaneously to the large ribosomal subunit in the absence of the $COB$ mRNA. These results indicate that binding of Cbp3 to the tunnel exit of the large ribosomal subunit causes a rearrangement of Cbs1 on the mitoribosome to the small subunit. This rearrangement would allow Cbs1 to place the $COB$ mRNA onto the small subunit for translation initiation. Our results could finally explain how Cbp3 triggers the initiation of cytochrome $b$ synthesis. Additionally, we provide for the first time evidence that translational activators play an active role in mitochondrial translation initiation as was suggested earlier (see Post-transcriptional regulation...
by translational activators). However, the role of Cbs2 in this process is not clear at the moment and will have to be studied in more detail.
specifika “translational activators” oberoende av COB mRNA. Sammanfattningsvis visade arbetet i denna doktorsavhandling hur den stora och varierande interaktionen av jästudoribosomen organiserar och reglerar mitokondriell translation. Dessa regleringsmekanismer visade många organellspecifika särdrag. Arbetet som presenteras här kommer att användas som utgångspunkt för att utveckla framtida studier som syftar till en bättre förståelse för hur mitokondrier är anpassade för att organisera genuttryck i organellen.
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