Mitochondrial translation and its impact on protein homeostasis and aging

Tamara Suhm

Abstract
Besides their famous role as powerhouse of the cell, mitochondria are also involved in many signaling processes and metabolism. Therefore, it is unsurprising that mitochondria are no isolated organelles but are in constant crosstalk with other parts of the cell. Due to the endosymbiotic origin of mitochondria, they still contain their own genome and gene expression machinery. The mitochondrial genome of yeast encodes eight proteins whereof seven are core subunits of the respiratory chain and ATP synthase. These subunits need to be assembled with subunits imported from the cytosol to ensure energy supply of the cell. Hence, coordination, timing and accuracy of mitochondrial gene expression is crucial for cellular energy production and homeostasis. Despite the central role of mitochondrial translation surprisingly little is known about the molecular mechanisms.

In this work, I used baker’s yeast *Saccharomyces cerevisiae* to study different aspects of mitochondrial translation. Exploiting the unique possibility to make directed modifications in the mitochondrial genome of yeast, I established a mitochondrial encoded GFP reporter. This reporter allows monitoring of mitochondrial translation with different detection methods and enables more detailed studies focusing on timing and regulation of mitochondrial translation. Furthermore, employing insights gained from bacterial translation, we showed that mitochondrial translation efficiency directly impacts on protein homeostasis of the cytoplasm and lifespan by affecting stress handling. Lastly, we provided first evidence that mitochondrial protein quality control happens at a very early stage directly after or during protein synthesis at the ribosome. Surveillance of protein synthesis and assembly into complexes is important to avoid accumulation of misfolded or unassembled respiratory chain subunits which would disturb mitochondrial function.

Keywords: mitochondrial ribosome, mitochondrial translation accuracy, mitochondrial communication, interorganellar communication, stress signaling, proteostasis, aging, yeast genetics, mitochondrial protein quality control, mitochondrial membrane protein insertion.
MITOCHONDRIAL TRANSLATION AND ITS IMPACT ON PROTEIN HOMEOSTASIS AND AGING

Tamara Suhm
Mitochondrial translation and its impact on protein homeostasis and aging

Tamara Suhm
Success consists of going from failure to failure without loss of enthusiasm.

- Winston Churchill -
List of publications


II. **Suhm T** and Ott M. Different genetic approaches to mutate the mitochondrial ribosomal protein S12. *Manuscript*


Additional publications

## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AAA</td>
<td>ATPase associated with diverse cellular activities</td>
</tr>
<tr>
<td>CLS</td>
<td>chronological lifespan</td>
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<tr>
<td>EF</td>
<td>elongation factor</td>
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<tr>
<td>eIF2α</td>
<td>eukaryotic initiation factor 2α</td>
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<tr>
<td>HSE</td>
<td>heat shock element</td>
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<td>HSR</td>
<td>heat shock response</td>
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<tr>
<td>IF</td>
<td>initiation factor</td>
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<tr>
<td>IMM</td>
<td>inner mitochondrial membrane</td>
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<tr>
<td>IPOD</td>
<td>insoluble protein deposit</td>
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<tr>
<td>IMS</td>
<td>intermembrane space</td>
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<tr>
<td>INQ</td>
<td>intracellular quality control compartment</td>
</tr>
<tr>
<td>IMiQ</td>
<td>intramitochondrial quality control compartment</td>
</tr>
<tr>
<td>IPTP</td>
<td>interorganellar proteostasis transcription program</td>
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<tr>
<td>MAGIC</td>
<td>mitochondria as guardian of cytosol</td>
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<tr>
<td>mitoCPR</td>
<td>mitochondrial comprised protein import response</td>
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<tr>
<td>mtDNA</td>
<td>mitochondrial DNA</td>
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<tr>
<td>MFRAT</td>
<td>mitochondrial free radical theory of aging</td>
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<tr>
<td>mPOS</td>
<td>mitochondrial precursor overaccumulation stress</td>
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<tr>
<td>MTS</td>
<td>mitochondrial targeting signal</td>
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<tr>
<td>NLS</td>
<td>nuclear localization signal</td>
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<tr>
<td>OMP</td>
<td>orotidine-5'-phosphate</td>
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<tr>
<td>OMM</td>
<td>outer mitochondrial membrane</td>
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<tr>
<td>OXPHOS</td>
<td>oxidative phosphorylation</td>
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<tr>
<td>PN</td>
<td>proteostasis network</td>
</tr>
<tr>
<td>Prx</td>
<td>peroxiredoxin</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>RF</td>
<td>release factor</td>
</tr>
<tr>
<td>RLS</td>
<td>replicative lifespan</td>
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<tr>
<td>RTG</td>
<td>retrograde response</td>
</tr>
<tr>
<td>RRF</td>
<td>ribosome recycling factor</td>
</tr>
<tr>
<td>STRE</td>
<td>stress element</td>
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<tr>
<td>UPR&lt;sub&gt;mt&lt;/sub&gt;</td>
<td>mitochondrial unfolded protein response</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>UPS</td>
<td>ubiquitin proteasome system</td>
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<tr>
<td>UTR</td>
<td>untranslated region</td>
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<tr>
<td>UPRam</td>
<td>UPR activated by mistargeted proteins</td>
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Introduction

Yeast as a model organism
In my work I used baker’s yeast *Saccharomyces cerevisiae* as a model organism. Yeast has many advantages for being used as a eukaryotic model organism including the number of tools accessible to manipulate its genome.

Basic yeast genetics
The complete genome of yeast was first sequenced and published in 1996 [1] and today it is the best annotated genome. It is relatively easy to create knock-outs or knock-ins by making use of the yeast’s own homologous recombination system [2]. There is even a commercially available deletion library in which each gene has been replaced by a kanamycin selection cassette. Using the kanamycin cassette to make knock-outs has the advantage that all auxotrophic markers are still available, e.g. to select for plasmids. However, knock-outs can also be made in the same way using auxotrophic selection cassettes. Laboratory yeast strains are modified in a way that they cannot synthesize all amino acids they need for growth and are therefore auxotroph for certain amino acids. Reintroducing the gene missing for biosynthesis of this amino acid concomitant with another protein modification of interest allows screening for positive transformation events by growth in the absence of this amino acid. Additional to a number of auxotrophic markers for positive selection there is the auxotrophic marker *URA3* which is a counter-selectable cassette, meaning that it can be selected for but also against the cassette [3]. In more detail, *URA3* encodes orotidine-5’-phosphate (OMP) decarboxylase which catalyzes a step in the *de novo* synthesis of uracil [4]. Therefore, cells expressing OMP decarboxylase can grow without uracil in the medium. However, in the presence of 5-fluoroorotic acid, yeast cells harboring the *URA3* cassette are not viable, as OMP decarboxylase will metabolize it to the toxic compound 5-fluorouracil [3]. 5-fluorouracil acts as thymidylate synthase inhibitor, thereby preventing thymidylate synthesis and eventually DNA replication.

A number of yeast plasmids are available containing different selection markers, promoters with various strength, as well as different multiple clon-
ing sites to make the cloning as easy and straight forward as possible. Additionally, there is the possibility to use integrative plasmids which lack an origin of replication and are therefore only propagated if integrated into the genome.

Yeast in mitochondria research

Being a facultative anaerobe organism makes yeast a very attractive model organism when studying mitochondrial processes. Facultative anaerobe describes the ability to survive without a functioning respiratory chain, as long as kept on the fermentative carbon source glucose. This ability of yeast can be used to study deletions of proteins of the respiratory chain, disease related mutations of respiratory chain proteins or mitochondrial gene expression proteins which often is not possible in mammalian cells [5]. Even the loss of mitochondrial DNA (mtDNA) (rho0) is not lethal to yeast as long as kept on a fermentative carbon source. A rho0 strain can be repopulated with mtDNA using cytoduction. This method allows the transfer of the mitochondrial genome between two strains [6, 7], while a mutation in one of the genes involved in karyogamy (kar1-1) slows down karyogamy [8] and prevents recombination of nuclear DNA. Ultimately, we can make directed alterations in the mitochondrial genome using biolistic transformation [9]. Besides plants, yeast is the only organism, so far, there this is possible. In order to translate a mRNA successfully on mitochondrial ribosomes it needs to be under control of 5’ and 3’ untranslated regions (UTR) [10-12]. The mitochondrial plasmid used in my studies encodes a part of endogenous COX2 as well as superfold GFP under control of 5’ and 3’ UTR of COX2 (Figure 1). A strain with an unfunctional COX2 (cox2-62) [13] is used as final recipient. By homologous recombination the plasmid is integrated at the COX2 locus giving rise to a functional COX2. Therefore, respiration can be used to screen for positive mitochondrial transformants. Thanks to the high mitochondrial recombination rate, yeast becomes homoplasmic (all mitochondrial DNA copies are identical) within a few generations [14, 15]. So far it is not possible to make directed alterations of the mtDNA in mammalian cells which is not due to the lack of trial [16, 17]. It might, however, become possible in the future allowing for more detailed studies of mitochondrial translation in higher eukaryotes.
Figure 1: Biolistic transformation. A plasmid encoding superfold GFP (sfGFP\textsuperscript{m}) flanked by 5’ and 3’UTR of COX2 as well as COX2 is transformed into cells lacking a functional COX2 (cox2-62). Eventually, mitochondrial homologous recombination gives rise to a new, fully functional, mitochondrial genome encoding sfGFP\textsuperscript{m} as a ninth protein (UTR, untranslated region; COX2, cytochrome c oxidase subunit 2; mtDNA, mitochondrial DNA) (figure copied from [18]).

Yeast as model for higher eukaryotes

Already in 1988 Botstein and Fink published an article in the journal Science about the promising future of yeast as an eukaryotic model organism and in 2011 they renewed this statement based on advances made in yeast research and the plethora of databases available [19, 20]. Many signaling pathways are conserved from the single cellular eukaryote yeast over Caenorhabditis elegans (C. elegans), Drosophila melanogaster and mice to humans. The first but not only example of functional conservation between yeast and mammals were the proto-oncogene proteins Ras1 and 2 [21]. Today it is clear that many pathways are conserved between humans and yeast and that without the great work in yeast many of these pathways would still be poorly characterized.

These pathways also include the ones which are implicated in aging. Studies performed in yeast gave insights into the aging process which was instrumental to uncover aging modulators in higher eukaryotes up to mice [22]. There are mainly two methods in yeast to study aging: chronological and replicative lifespan (CLS and RLS, respectively) (Figure 2) [22, 23]. In chronological aging, survival of non-dividing cells is monitored. In detail, cells are grown to stationary phase where they enter a quiescent-like state. By plating on rich media, the ability of the cells to escape this quiescent-like state is studied. In replicative aging, the amount of cell divisions of a single mother cell is observed. It was argued that RLS is a model which replicates mitotically active cells as stem cells in humans, while CLS more relates to
post-mitotic cells. However, it was shown that genes involved in yeast RLS show a significant overlap with worm aging (post-mitotic organism). Furthermore, both methods played an important role in discovering the role of the conserved Ras-PKA and TOR-Sch9 signaling pathways in aging [23].

Figure 2: Replicative and chronological lifespan. In replicative lifespan the number of cell divisions of a single mother cell is followed (A). In chronological lifespan a liquid culture is grown to stationary phase and the ability to escape a quiescent-like state is studied (B) (d, daughter cell; m, mother cell).
Mitochondria

General functions
Mitochondria are surrounded by a double membrane subdividing the organelle into four compartments: the outer mitochondrial membrane (OMM), the intermembrane space (IMS), the inner mitochondrial membrane (IMM) and the matrix. Mitochondria are most famous for being the powerhouse of the cell, producing most of the cell’s ATP.

![Mitochondrial diagram](image)

**Figure 3: Mitochondrial functions.** Besides their well-known role as powerhouse of the cell, mitochondria are involved in other processes including metabolic reactions as TCA cycle, fatty acid oxidation, amino acid metabolism, cell signaling by maintaining calcium homeostasis, ATP/ADP ratio and apoptosis as well as iron sulfur cluster biogenesis and heme biosynthesis (TCA, tricarboxylic acid; ANT, adenine nucleotide translocase; OXPHOS, oxidative phosphorylation; ROS, reactive oxygen species).

The oxidative phosphorylation (OXPHOS) system, which is located in the IMM, consists of three respiratory complexes in yeast (complex II, III and IV) as well as the ATP synthase (complex V). Electrons are transferred from NADH and FADH$_2$, which were reduced in the TCA cycle, through the respiratory complexes to eventually reduce oxygen to water. Complex III and IV use the energy released by the transport of electrons from a donor to a more electronegative acceptor to translocate protons from the matrix site to the IMS. The resulting electrochemical gradient is used by the ATP synthase
to phosphorylate ADP to ATP. Besides this crucial role, mitochondria also fulfill a number of other important tasks like heme biosynthesis, iron-sulfur cluster biogenesis, calcium storage and signaling, harboring many metabolic pathways at least partly and playing an important role in apoptosis (Figure 3) [24, 25]. About 1.5 billion years ago, mitochondria originated by endosymbiosis of an α-proteobacterium with an ancestral eukaryotic cell [26]. During evolution, mitochondria transferred most of its DNA to the nucleus [27]. Nevertheless, the organelle still contains its own small genome and gene expression machinery (Figure 4). In yeast, mtDNA encodes for two rRNAs, 24 tRNAs and eight proteins, whereof seven are membrane proteins and subunits of the OXPHOS system [28]. However, most of the about 1000 mitochondrial proteins are encoded in the nucleus, transcribed there, translated on cytosolic ribosomes and imported into mitochondria (Figure 4) [29, 30].

**Figure 4: Dual genetic origin of OXPHOS system.** Mitochondrial DNA encodes seven subunits of the OXPHOS, while the remaining subunits are encoded in the nuclear genome, translated in the cytosol and imported into mitochondria (OXPHOS, oxidative phosphorylation; mtDNA, mitochondrial DNA; TOM, translocase of outer membrane; TIM, translocase of inner membrane).

The high hydrophobicity of mitochondrial encoded proteins could be one reason why mitochondria kept these genes. It was shown that a nuclear encoded version of cytochrome b could not be imported into mitochondria [31], while Atp6 expressed from the nuclear genome could be imported though not very efficiently [32]. Another plausible reason is the different codon usage of mitochondria. Lastly, it might be an advantage to directly
couple assembly to synthesis in the organelle. Even though there are several nonexclusive explanations why mitochondria kept this small number of genes, it is still questioned if this is worth the costs as there are roughly 250 proteins involved in maintaining and expressing the mitochondrial genome [33].

As the OXPHOS system, as well as the mitochondrial ribosome, consist of subunits of dual genetic origin, the two gene expression systems need to be coordinated to allow for proper assembly of these macromolecular complexes ensuring ATP production. This coordination will be discussed in the ‘Mitochondrial translation’ chapter.

Mitochondria and aging – what is the connection?

Mitochondrial dysfunction is a hallmark of aging [34]. In many model organisms as well as in humans an increase in mtDNA mutations was observed during aging [35-37] as well as an overall decline in mitochondrial function [38] and respiratory chain function [36, 37, 39].

Already in 1956, Harman postulated the free radical theory of aging. This theory describes how an increased production of reactive oxygen species (ROS) over time attacks DNA, proteins and lipids in the cell [40]. As the respiratory chain, located in the IMM, is the major site of ROS production, it was later extended to the mitochondrial free radical theory of aging (MFRAT) (Figure 5) [41]. Being the main site of ROS production, mitochondria, especially mtDNA, were thought to be the primary site of ROS damage. Damage to mtDNA gives rise to mutated versions of respiratory chain proteins which in turn result in an even higher ROS leakage thereby entering a vicious cycle of ROS production and mtDNA mutations.

In favor of the MFRAT, many studies show a clear involvement of ROS in aging phenotypes. In contrast, others show that an increase in ROS can even prolong lifespan [37, 42, 43] and that an increased oxidative defense system alone is not sufficient to prolong lifespan [37, 44-49]. The involvement of ROS in aging is still a highly debated topic and most likely the dosage makes the poison.

One of the main studies challenging the MFRAT is the mutator mouse, which accumulates mtDNA mutations due to a mutation in the proofreading function of the mitochondrial polymerase Polg. However, this increase in mtDNA mutations which was accompanied by an early onset of aging phenotypes, did not correlate with an increase in oxidative stress even though there was mitochondrial dysfunction reported [50, 51]. Additionally, the increase in mtDNA mutations observed in the mutator mouse showed a rather linear increase in ROS during adulthood and not an exponential increase as it would be expected in the postulated vicious cycle of the MFRAT [51].
Even though the mutator mouse nicely shows the first causative link between mtDNA mutations and early onset of aging some limitations have to be kept in mind. In normal aging tissue, the number of mtDNA mutations is much lower than what was observed in the mutator mouse and it is not clear how they account for aging [52]. Even in the heterozygous mutator mouse, the number of mutations acquired is higher than in humans, yet this mouse did not show an aging phenotype [53]. Furthermore, the mutations in the mutator mouse are point mutations, while in elderly humans mtDNA deletions dominate [54, 55]. Such deletions were replicated by the deletor mouse which contains patient-mutations in the mtDNA helicase TWINKEL [56]. The deletor mouse, however, did not show an early onset of aging despite mtDNA deletions and respiratory chain dysfunction.

In contrast to MFRAT, the error-catastrophe theory of aging postulated that a decrease in the accuracy of translation drives aging [57]. Experiments testing this hypothesis were not able to show a relationship between translation accuracy and aging [58]. However, these experiments focused on cytosolic ribosomes while a study focusing on mitochondrial ribosomes in yeast showed that increasing mitochondrial translation accuracy with the drug erythromycin increased RLS [59]. Also in bacteria, the ancestor of mitochondria, a decrease in translation fidelity during cell cycle arrest was reported [60, 61].

Making a long story short, the involvement of ROS in aging is still debated and the MFRAT has been largely refuted. Nowadays it is rather widely assumed that mtDNA mutations caused by replication errors correlate with the aging phenotype in humans [62]. The involvement of mitochondrial dysfunction in aging, however, is clear but the complex nature of mitochondria and its involvement in so many crucial functions makes it difficult to disentangle cause and consequence [55].

Figure 5: Mitochondrial free radical theory of aging. ROS leakage from OXPHOS damages mtDNA over time. mtDNA mutations give rise to defective OXPHOS proteins causing more ROS leakage and entering a vicious cycle which drives aging (mtDNA, mitochondrial DNA; OXPHOS, oxidative phosphorylation; ROS, reactive oxygen species).
Translation

Bacterial translation

An overview
In bacteria, translation was and is extensively studied and a detailed picture of the single steps was built over the last decades. Translation can be divided into four steps: initiation, elongation, termination and recycling. These steps will be briefly described in the following paragraph. For a more detailed review see Rodnina 2018 [63].

In a non-translating state, initiation factor 3 (IF3) binds to the small subunit of the ribosome to prevent its premature association with the large subunit. During initiation the small ribosomal subunit together with IF1, 2 and the fMet-tRNA^{fMet} binds the mRNA. The tRNA binding displaces IF3 which in turn allows the large subunit of the ribosome to join. Subsequent GTP hydrolysis by IF2 releases the initiation factors. The ribosome is now ready for translation.

The ternary complex, consisting of aminoacyl-tRNA together with elongation factor Tu and GTP (EF-Tu-GTP), first makes codon independent interactions with the ribosome, followed by codon recognition to start the first round of elongation. In most cases, cognate and near-cognate tRNAs only differ in one base. Therefore, the difference in free energy released by codon recognition between cognate and near-cognate tRNA is not enough to reach the high accuracy of $10^{-3}$ to $10^{-4}$ [64, 65] which is observed in living cells. Instead, two independent kinetic proofreading steps, which are separated by GTP hydrolysis, are required [66-68]. In a first step, initial selection can lead to rejection of non-cognate tRNAs in complex with EF-Tu from the ribosome. Non-cognate tRNAs are rejected with higher probability than cognate ones. After codon-anticodon interaction and GTP hydrolysis by EF-Tu, the tRNA can either be rejected in a second proofreading step or accommodated in the A site (Figure 6). The recognition of the cognate tRNA induces a conformational change in the small subunit referred to as domain closure (see section ‘Domain closure model during decoding’). Once the cognate tRNA is accommodated in the A site, peptide bond formation is catalyzed by the peptidyl transferase center of the large ribosomal subunit leaving a dipeptidyl tRNA in the A site and a deacetylated tRNA in the P site. Thereafter, the mRNA and tRNA translocate through the ribosome with help of elongation factor G (EF-G), another GTPase. This places the dipeptidyl tRNA in the P site, while leaving the A site with the new codon.

Once the ribosome encounters a stop codon translation is terminated. There are three different stop codons in bacteria: UAA, UGA and UAG. Depend-
ing on the stop codon release factor 1 or 2 (RF1 or 2) bind. They hydrolyze the ester bond, by which the peptide is bound to the tRNA, to release the peptide. RF3 then catalyzes the dissociation of RF leaving the ribosome with bound tRNA and mRNA.

In order to initiate a new round of translation, ribosome recycling is necessary. The ribosome is split by ribosome recycling factor (RRF) and EF-G. IF3 binding releases the tRNA while the mRNA dissociates spontaneously [63, 69].

![Diagram of translation process]

**Figure 6: Two kinetic proofreading steps to select cognate tRNA.** During initial selection the aminoacyl-tRNA interacts codon independent with the ribosome. Subsequent codon-anticodon interaction activates GTP hydrolysis and induces domain closure. In a second proofreading step the aminoacyl tRNA is rejected or accommodated in the A-site followed by peptide bond formation (figure copied from [70]).

**Domain closure model during decoding**

The decoding center of the ribosome is highly conserved and is located in the small subunit of the ribosome [71, 72]. It consists of the bases G530 (in helix18) as well as A1492 and A1493 (in helix 44) of the 16S rRNA in bacteria [73]. The domain closure model, established by the Ramakrishnan group [74, 75], describes how the ribosome discriminates cognate tRNAs from near-cognate tRNAs. This model suggests that only if the cognate tRNA is placed in the A site, a conformational change of the ribosome is triggered.

In detail, A1492 and A1493 flip outwards towards the codon anticodon helix establishing interactions with the first and second position of the codon. Additionally, G530 switches from syn to anti conformation and contacts the second and third position of the codon. These small conformational changes trigger a larger change in the small subunit by rotation of the head towards the subunit interface and the shoulder towards the intersubunit space.

During this conformational change hydrogen bonds between lysine residues in S12 and the phosphate backbone in helix 27 and 44 of the 16S rRNA are formed establishing new contacts between the 16S rRNA and the ribosomal
protein S12. Furthermore, contacts between the ribosomal proteins S4 and S5 are broken. 
Summed up, this model suggests that the ribosome is in an open form and undergoes rearrangements to a closed form only when the cognate tRNA has bound. This model was based on structures of the small subunit with bound A site tRNA anticodon stem loop (ASL) and oligonucleotide for mRNA. Today, this model for discrimination between cognate and near-cognate tRNA is debated in the ribosome field. There are structures of the 70S ribosome with long mRNA and natural tRNA available. These structures showed identical rearrangements of the small subunit irrespective whether the bound tRNA was cognate or near-cognate [76-78].

**Changed translation accuracy by aminoglycosides and mutations**

Aminoglycosides are a class of antibiotics which decrease translation accuracy by binding to the small subunit of the ribosome. Streptomycin for example binds to the small subunit at the decoding center [79]. It was proposed to stabilize the closed form of the small subunit resulting in an error-prone translation. Paromomycin, another aminoglycoside, binds to helix 44 of the 16S rRNA. It was shown to induce domain closure which normally requires the presence of cognate tRNA and proper codon-anticodon interaction [74, 80]. It leads to non-specific binding of tRNA to the A site, increases GTPase activation rate of near-cognate codon-anticodon interactions, thereby decreasing dissociation rate and increasing peptide bond formation [80].

Mutations affecting translation fidelity were first found by hypersensitivity of strains towards the aminoglycoside streptomycin. These strains contained mutations in the ribosomal proteins S4 and S5 [81]. It was suggested that these mutations facilitate the breakage of polar interactions between the two proteins which in turn induces domain closure and causes error-prone translation [74, 79, 82]. This model was, however, questioned and criticized as oversimplified due to the observation that not all mutations affecting fidelity were necessarily located at the interface of S4 and S5 or destabilize their interaction [83, 84].

The ribosomal protein S12 has a unique position in the bacterial ribosome. It contacts helices 18, 27 and 44 [85] of the 16S rRNA which makes it the only protein in such close vicinity to the decoding center. S12 helps to orient several 16S rRNA residues for codon-anticodon base pairing. Mutations in the ribosomal protein S12 showed resistance or hypersensitivity towards streptomycin [86-89]. Also here it is assumed that the phenotypes are caused by the interference of the mutations with domain closure [74]. Mutations which cause a streptomycin resistant phenotype are often in lysine residues which are involved in salt bridge formation during domain closure. It is likely that
by mutating these residues and thereby destabilizing the salt bridges, domain closure is hindered resulting in a hyperaccurate translation.

Mitochondrial translation

An overview

Because of the endosymbiosis theory for mitochondrial origin it was assumed that the translation machinery is highly conserved between bacteria and mitochondria. As known today, yeast mitochondrial ribosomes have a higher content of proteins than rRNA compared to their bacterial counterparts and this difference is even more pronounced in mammalian mitochondrial ribosomes [90]. Moreover, recent advances in cryo-electron microscopy showed that the structure of the ribosome did change remarkably during evolution [71, 91]. Besides the increased protein content, mitochondrial ribosomes also do not contain a 5S rRNA [92]. However, certain parts of the ribosome are highly conserved, like the decoding center (in the small ribosomal subunit) and the peptidyl transferase center (in the large ribosomal subunit) [71, 72, 90, 92]. Also the susceptibility towards aminoglycosides has not changed, which explains the side effects caused by certain antibiotics when used as drugs [93].

Another key component of translation is the mRNA, which also differs between bacteria and mitochondria, as mitochondrial mRNAs do not contain a Shine-Dalgarno sequence. In bacteria, this sequence in the 5’ untranslated region (UTR) assures proper initiation by pairing with bases in the 16S rRNA and positioning of the start codon in the ribosomal P site. Although in yeast mitochondria, the mRNA contains a 5’UTR, it does not contain a Shine-Dalgarno sequence, while mammalian mitochondrial mRNAs do not contain a 5’UTR at all [92].

In yeast, instead, a set of proteins called translational activators has been described to interact with the 5’UTR and the ribosome to initiate and coordinate translation in mitochondria [94, 95]. So far only one translational activator has been identified in mammalian mitochondria (TACO1) [96]. Nevertheless, the general steps of translation (initiation, elongation, termination and recycling) are conserved. There are homologs for IF2 and IF3. IF1 is missing, but its role may be played by an extra domain in IF2. Also the delivery of the fMet-tRNA\textsuperscript{fMet} (in mammalian mitochondria the Met is not formylated) and joining of the large subunit are similar as in bacteria [92].

The most conserved step is elongation where homologs of EF-Tu, EF-G and EF-Ts (not in yeast) are present. Due to a reduced number of codons encoding a stop in mitochondria (only UAA and UAG, UGA encodes tryptophan), there is only a homolog for the release factor RF1. A homolog of the recy-
clinging factor RRF eventually splits the ribosome into subunits. In contrast to bacteria, there are two EF-G homologs, one involved in translocation during elongation while the other one assists RRF1 during recycling [92].

**Accuracy of translation**

As described above, bacterial translation is highly accurate with an error frequency of $10^{-3}$-$10^{-4}$. To date, not much is known about accuracy of mitochondrial translation, as there is neither a robust *in vitro* translation system nor *in vivo* reporters. One fact that is however known, is that mitochondrial translation accuracy is important for life. First of all, proteins encoded by the mitochondrial genome are core subunits of the respiratory chain. An inaccurate mitochondrial translation, therefore, gives rise to malfunctioning or unassembled respiratory chain complexes and eventually low levels of ATP as well as increased rates of ROS production [97].

Secondly, severe mitochondrial diseases are caused by mutations in mitochondrial tRNAs. The disease MELAS, for example, is caused by a mutation in Leu-tRNA which causes a decrease in translation accuracy [98]. Furthermore, the A1555G mutation in 12S rRNA of human mitochondrial ribosome causes aminoglycoside hypersensitivity and stress-induced deafness. In a study with mitochondria-bacteria-hybrid ribosomes, this mutation was also shown to decrease translation accuracy [99]. Another study performed in yeast, showed an increased lifespan when translation accuracy was increased by the antibiotic erythromycin [59].

As described in the chapter ‘Bacterial translation’, there are mainly three ribosomal proteins involved in the decoding step in bacteria, namely S4, S5 and S12. These proteins have homologs in mitochondria (Nam9, Mrps5 and Mrps12 respectively) (Table 1). Also the recent high resolution ribosome structures showed conservation of the decoding center [71, 92]. Therefore, the assumption that mutations in Nam9, Mrps5 or Mrsp12 would affect translation in a similar way as their bacterial counterparts is eligible. Indeed, a recent study showed that a mutation in Mrps5 decreases mitochondrial translation accuracy and alters stress- and age-related behavior in mice [100]. An older study discovered a mutation in Nam9 which suppresses an ochre mutation in COX2. The authors speculate that the premature stop codon in the mutated version of COX2 is read through by decreased translation accuracy in mitochondria due to the Nam9 mutation [101].

**Table 1: Orthologues of ribosomal proteins in bacteria, yeast and mammals.**

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Yeast</th>
<th>Mammals</th>
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<tbody>
<tr>
<td>S4</td>
<td>Nam9</td>
<td>Mrps4</td>
</tr>
<tr>
<td>S5</td>
<td>Mrps5</td>
<td>Mrps5</td>
</tr>
<tr>
<td>S12</td>
<td>Mrps12</td>
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Monitoring mitochondrial translation

Mitochondrial translation can be followed by incorporation of radioactive methionine ($^{35}$S) into newly translated proteins, followed by separation of the translation products on SDS-PAGE and subsequent visualization by autoradiography [102-104]. One hurdle, however, is to circumvent labeling of cytosolic proteins. There are two ways to address this problem. Cytosolic translation can be inhibited by cycloheximide, an antibiotic which blocks the translocation step of elongation. This prevents labeling of cytosolic proteins but also comes with a number of side effects. Inhibition of protein synthesis leads to an increase in free amino acids which activates TOR signaling [105-107], thereby causing changes in cell growth, nutrient signaling and life span [108-110]. Furthermore, a recent study showed that cycloheximide directly alters mitochondrial protein synthesis within less than 15 minutes [111]. Lastly, not only protein synthesis but also protein degradation is altered by cycloheximide [112]. Taken together, these effects severely impact on cellular homeostasis and do not reflect a physiological scenario.

Alternatively, the labeling can be performed in isolated organelles. This system certainly enables visualization of merely mitochondrial proteins but also lacks all the signals coming from the cytosol or nucleus which were shown to impact on mitochondrial translation [95, 110, 111].

Taken together, both methods enable visualization of protein synthesis in mitochondria but do not reflect a physiological scenario and, therefore, are not appropriate to study activation, timing or regulation of translation in the context of cell physiology. Another approach by Fox and coworkers was integration of GFP into the mitochondrial genome. Following GFP expression allows readout of mitochondrial translation in vivo but as the GFP replaced the open reading frame of COX3 in the mitochondrial genome it led to non-respiring cells which prevented physiological studies [113].

Co-translational membrane insertion of mitochondrial encoded proteins

Most proteins produced by the mitochondrial ribosome are highly hydrophobic membrane proteins and need to be inserted into the membrane to prevent aggregation. This task is facilitated by membrane attachment of the mitochondrial ribosome [92, 94, 114]. In mammalian mitochondria, the ribosome is anchored to the membrane via Mrpl45, a ribosomal protein of the large subunit [92]. Mba1, the yeast homolog of Mrpl45, is a peripheral membrane protein which was shown to interact with the ribosome near the exit tunnel in close proximity to the nascent chain [115, 116]. Another membrane protein interacting with the tunnel exit as well as the nascent chain is the insertase Oxa1. Oxa1 is part of the Oxa1/YidC/Alb3 protein family and inserts mitochondrial proteins into the IMM even though it is not absolutely essential for membrane protein insertion [115, 117, 118]. A double deletion of Oxa1 and Mba1, however, showed impaired membrane insertion [115]. A third mem-
brane protein interacting with the large subunit is Mdm38. Its mammalian homolog is involved in neurodegeneration. A recent study identified Mrx15 as a new ribosome receptor and showed overlapping functions of Mrx15 and Mba1 in co-translational protein membrane insertion [119]. Together these factors mediate mitochondrial ribosome membrane attachment and co-translational membrane insertion of proteins [94].

**Coordination of mitochondrial and nuclear translation**

Assembly of a functioning OXPHOS system is crucial for aerobic life and requires coordination of mitochondrial and nuclear gene expression. In a recent study, Couvillion *et al* applied a ribosome profiling approach to study mitochondrial as well as cytosolic translation upon a metabolic switch from fermentation to respiration in yeast [111]. They reported that mitochondrial as well as cytosolic translation of OXPHOS subunits is regulated in a fast and coordinated fashion.

Furthermore, translational activators are known to play an important role in coordination of translation and assembly of OXPHOS complexes in yeast mitochondria [95]. It was shown that translational activators can also act as chaperones which can be sequestered in assembly intermediates and only upon proper assembly of the complex are released to start a new round of translation activation. Thus, mitochondrial translation is leveled to the number of nuclear-encoded subunits available.

In mammalian mitochondria only one translational activator has been discovered so far. A different concept, however, was described for translational plasticity in mammalian mitochondria. It was shown that for complex IV assembly, mitochondrial translation is adjusted to fit the amount of nuclear-encoded subunits [120]. In Cox4 depleted cells, *COXI* translation is halted and the stalled ribosomes, containing the Cox1 peptide partially inserted into the membrane, interact with assembly intermediates.

This shows that mitochondrial translation is regulated in one or another way to match the availability of nuclear-encoded subunits in yeast as well as mammals, albeit with distinct mechanisms.
Protein Quality Control

Protein quality control in the cytosol
The happiness of every cell depends on proper functioning proteins. This is achieved by the constant surveillance of de novo protein folding as well as protein refolding and degradation of damaged or unfolded proteins to maintain protein homeostasis (proteostasis). The system responsible is called proteostasis network (PN) and consists of molecular chaperones and co-chaperones as well as the degradation pathways autophagy and ubiquitin-proteasome system (UPS). Also the active sequestration of aggregates into specific compartments inside the cell is part of the PN. In parallel to the PN, adaptive transcription programs are turned on to restore proteostasis during stress.

Molecular chaperones
There are five classes of ATP-dependent chaperones: Hsp70, Hsp40, Hsp90, Hsp60 (chaperonin) and Hsp100. Moreover, there are the ATP-independent small heat shock proteins (sHsp) (Figure 7) [121, 122].
Hsp70 can act on the nascent polypeptide emerging from the ribosome to ensure proper folding as well as on misfolded proteins. It targets the proteins for degradation or reactivation by recognizing and binding exposed hydrophobic segments. Hsp40 (J-proteins) interacts with Hsp70 to accelerate its ATPase activity and is responsible for Hsp70 substrate specificity. Additionally, Hsp70 interacts with nucleotide exchange factors (NEF) [123, 124].
Hsp90 functions downstream of Hsp70, where it mainly assist in final folding and assembly of higher order complexes [123, 125].
Hsp60 also acts downstream of Hsp70 and facilitates final folding of proteins. Hsp60 forms multimeric complexes of cylindric shape building a folding chamber for its client proteins [122, 126].
Despite all these surveillance mechanisms for protein folding and refolding, the proteostatic capacity can become overwhelmed by stress or aging, causing aggregate formation [125, 127]. For a long time, it was assumed that proteins accumulated in aggregates cannot be reactivated. It was only in the 1990s when Lindquist and co-workers showed that Hsp104, a protein of the Clp/Hsp100 protein family, can disaggregate proteins in yeast [128]. As many other chaperones and proteases, Hsp104 and its bacterial homolog ClpB are members of the AAA+ (ATPase associated with diverse cellular activities) superfamily [123, 129]. Homologs of the AAA+ superfamily are found in fungi, bacteria, plants and mitochondria of eukaryotes. As the name suggests, members of the AAA+ superfamily fulfill many different cellular functions but share a highly conserved 200 to 250 amino acid long ATPase module. This module contains the Walker A and B motifs, which harbor the
nucleotide binding and hydrolysis domain. Another similarity amongst the members is the assembly into hexameric ring structures [130]. Hsp104/ClpB mostly act in a bi-chaperone system together with Hsp70/DnaK [129, 131]. First, Hsp40/DnaJ binds to the aggregated proteins ensuring substrate specificity of Hsp70/DnaK. Once Hsp70/DnaK bound to the hydrophobic patches of the aggregates, it recruits Hsp104/ClpB. Hsp104/ClpB subsequently unfolds the aggregates [127, 132, 133]. Metazoans lack a homolog of Hsp104/ClpB in the cytosol but they still show disaggregase activity [121, 125]. It was recently reported that this disaggregase activity comes from a concerted action of Hsp70, Hsp40 and Hsp110 (NEF of Hsp70) [134-136]. Following unfolding, the protein is either refolded assisted by Hsp70/DnaK or channeled to the degradation pathway. It is still not exactly clear how the triage decision between refolding, degradation and sequestration is achieved on a molecular level [125].

**Figure 7: Proteostasis network.** Hsp70 assists proteins emerging from the ribosome in folding. Interaction with Hsp40 confers substrate specificity. It also interacts with misfolded proteins helping them to refold or channel them to the UPS for degradation. Hsp90 and Hsp60 act downstream of Hsp70 and assist in final folding as well as complex assembly. In a bi-chaperone system with Hsp104, Hsp70 helps disaggregating proteins (NEF, nucleotide exchange factor; UPS, ubiquitin proteasome system).

**The ubiquitin-proteasome system**

The ubiquitin-proteasome system (UPS) mediates degradation of misfolded or unfunctional proteins as well as proteins which are no longer needed. The first step to mark a protein for degradation is ubiquitination. Ubiquitin is transferred to the protein via the concerted actions of three enzymes, E1, E2
and E3. The ubiquitin-activating enzyme E1 forms an energy rich thioester with ubiquitin. Following activation, ubiquitin is transferred to the ubiquitin-conjugating enzyme E2 and, subsequently, the ubiquitin-ligase E3 transfers ubiquitin to a lysine residue within the target protein. E3 is also the enzyme which confers substrate specificity. This process can be reversed by deubiquitinating enzymes. Ubiquitinated proteins are recognized and degraded by the proteasome.

The 26S proteasome consist of the 20S core particle and 19S regulatory particles. The 19S particle, a member of the AAA+ superfamily, recognizes the substrate and uses energy derived from ATP hydrolysis to transfer it into the proteolytic chamber of the 20S particle [137, 138]. Degradation of proteins via the UPS not only determines the proteome of the cytosol but also of other organelles.

**Sequestration compartments**

Another arm of the PN is the sequestration of aggregated proteins into separated compartments. Besides stress-induced aggregation, aggregates can also form during the normal life of a cell. Aggregation under non-stress conditions can be caused by mutated versions of proteins, inefficient protein biogenesis or accumulation of unassembled complex subunits [125, 139]. At first aggregates form stochastically within the cytosol but are then actively collected at specific sequestration sites [122, 140].

In yeast, there are three different aggregate structures described: the intracellular quality control compartment (INQ, former JUNQ), the insoluble protein deposit (IPOD) and CytoQ (also stress foci/Q-bodies).

INQ contains ubiquitinated proteins and was shown, by cryo-electron microscopy, to be localized inside the nucleus [141]. It only builds up during stress and disappears after stress release. The clearance of INQ probably happens by either refolding with the help of the bi-chaperone system or via degradation through the colocalized proteasome [142].

IPOD, which in contrast to INQ, is a more rigid structure of immobile, not ubiquitinated proteins, can also build up in unstressed cells. It colocalizes with the autophagy marker Atg8 [142].

The third compartment, CytoQ, is the equivalent to INQ but is localized in the cytosol. In yeast, for example, Hsp42, a small Hsp, colocalizes with several small stress foci which then merge to a single aggregate [125, 140, 142, 143].

In mammals, the formation of similar compartments was reported, pointing towards an evolutionary conservation of this process [142, 144]. What might be the advantage to sequester aggregates in specific compartments? Sequestration of aggregates separates the aggregates from cellular processes, lowers the load on the protein quality control system, increases refolding/degradation rates and allows asymmetric inheritance of aggregates. All this converges in the reduction of cytotoxicity [125, 140].
**Hsf1 and Msn2/4 - adaptive responses**

In parallel to the PN handling the misfolded proteins during stress, the heat shock response (HSR) is turned on [122, 126]. This adaptive response results in a decreased overall translation rate to lower the burden on the PN, while chaperones and the proteolytic system are upregulated.

Figure 8: Transcriptional programs upon stress in yeast. In yeast, Hsf1 is always in the nucleus and bound to HSE in promoters of target genes but upon stress its activity is increased by hyperphosphorylation. The transcription factors Msn2/4 are phosphorylated by the nutrient sensitive kinases TOR and PKA and anchored in the cytosol via interaction with Bmh2. Upon stress, PKA and TOR are inhibited and Msn2/4 migrate to the nucleus where they bind to STRE elements in promoters of target genes (Hsf1, heat shock factor 1; PKA, protein kinase A; TOR, target of rapamycin; HSE, heat shock element; STRE, stress responsive element).

In unstressed mammalian cells the transcription factor heat shock factor 1 (Hsf1) is bound by chaperones as an inactive monomer in the cytosol. Upon stress, the chaperones are titrated away to help in protein refolding leaving a free form of Hsf1 [145, 146]. Free Hsf1 then forms trimers and relocates to the nucleus where it induces transcription of PN components by binding to heat shock elements (HSE) in their promoter [145, 146]. Additionally, Hsf1 is regulated by post-translational modifications [147, 148]. In contrast to mammals, Hsf1 in yeast is always localized in the nucleus and active, but its activity is increased by hyperphosphorylation during stress (Figure 8) [123].

Another adaptive response is orchestrated by the transcription factors Msn2/4. Msn2/4 are zinc-finger transcription factors activating the general stress response in yeast [149, 150]. Under non-stress conditions, the nutrient sensitive signaling pathways TOR and cAMP/PKA inhibit Msn2/4 activity by phosphorylation and anchoring it in the cytosol via interaction with the 14-3-3 protein Bmh2 [151]. Upon stress, Msn2/4 translocate to the nucleus.
where they bind to stress elements (STRE) in promoters of target genes, activating transcription and thereby regulating protein synthesis, cell growth, stress resistance and metabolism (Figure 8) [110, 152, 153]. Often, but not always, STRE and HSE are found in promoters of the same target genes. A few years back, for example, it was shown that they can act together as well as independently to activate HSP104 transcription [154].

**Peroxiredoxins**

Peroxiredoxins (Prx) are a conserved class of antioxidative enzymes, containing a peroxidatic cysteine residue in their catalytic site. This cysteine (-SH) gets oxidized to sulfenic acid (-SOH) during the catalytic cycle, followed by disulfide formation (S-S) with a second cysteine, resulting in a dimer. Reduction of the disulfide by thioredoxin (Trx) completes the catalytic cycle of Prx. Alternatively, the Prx-SOH can become hyperoxidized to sulfonate (-SOOH) (Figure 9). This represents an inactive, locked form of Prx which can be reduced to Prx-SOH by sulfiredoxins (Srx) [155-157].

![Figure 9: Peroxiredoxins in yeast.](image)

Tsa1-SH, the major peroxiredoxin in yeast, gets hyperoxidized to Tsa1-SOOH. The enzymes Srx1 and Trx1 reduce it again to Tsa1-SH (figure copied from [158]).

As the route of Prx hyperoxidation and inactivation is evolutionary conserved, it was concluded that it must be accompanied with a gain-of-function. Indeed, it was shown that this form of Prx not only forms high molecular-weight complexes but also has chaperone function. Furthermore, the floodgate model was proposed. In this model, Prx is inactivated in order to allow a local increase in peroxide levels which enables peroxide to act as a local signaling molecule without disturbing the cells redox state [156, 157]. The function of Prx in aging is currently under investigation. It was shown that during aging, yeast as well as rat Prx become hyperoxidized [159, 160] and that an extra copy of Srx1 prolongs lifespan in yeast [159]. Additionally, Hanzén *et al* showed that the hyperoxidized form of Tsa1 (the major Prx in yeast) is needed for H$_2$O$_2$- and aging-induced aggregate recognition by the bi-chaperone system Hsp70/Hsp104 and that the clearance of these aggregates is dependent on Tsa1 as well as Srx1 [161].
Proteostasis during aging
Loss of proteostasis is a hallmark of aging [34]. It was shown in model organisms ranging from yeast to mice as well as in humans, that the proteostatic capacity declines with age and aggregates accumulate [139, 162, 163]. The significance of keeping proteostasis is further shown by the number of age-related diseases caused by aggregates, such as Parkinson’s or Alzheimer’s disease [126, 139]. Several studies also reported that deletion of components of the PN resulted in a shortened RLS in yeast accompanied by accumulation of aggregates [159, 164, 165]. It is assumed that during aging the capacity of the PN declines due to errors in translation or splicing and accumulation of oxidatively damaged proteins. This, of course, would also affect proteins of the PN, especially oxidation-prone chaperones [139]. The sequestration of aging-induced aggregates is similar as during acute stress but the sequestration sites do neither correspond to INQ nor IPOD. With increasing age, however, the ability of the cell to sequester aggregates decreases, probably due to a breakdown of different cellular functions [139]. This leaves the cell with multiple cytotoxic aggregates.

Protein quality control inside mitochondria
Mitochondria harbor many crucial processes for cell survival (see chapter ‘Mitochondria’). Therefore, it is not surprising that mitochondrial protein homeostasis is involved in cellular fitness and mitochondrial dysfunction is associated with many, but especially neurological, diseases as well as aging. Hence, it is an important task of the organelle to keep its proteins in a functional state. In order to do so, mitochondria contain their own proteostasis network which closely resembles the one of their bacterial ancestor [166, 167]. They contain a complete set of chaperones as well as proteases (Figure 10) [167, 168]. In a second line of defense, damaged mitochondria can be eliminated by mitophagy [168].

Mitochondrial chaperones
Mitochondria contain the same classes of chaperones as are found in bacteria and the cytoplasm of eukaryotes. Members of the Hsp70 family, Hsp40 family, Hsp60 family as well as Hsp100/ClpB family have been found and studied inside the organelle [167, 168]. The major mitochondrial Hsp70 in yeast is Ssc1. Ssc1 assists in two important events: import of proteins into mitochondria as well as folding of polypeptides [169]. It was shown to interact with Tim44 [170], a protein of the import machinery, and is necessary for proper import of proteins into the matrix. After import, Ssc1 is also the first chaperone to interact with the newly imported, unfolded protein. As other Hsp70s, Ssc1 binds to hydrophobic patches in the protein and assists in folding. It also helps mitochon-
drial encoded proteins to fold once they emerge from the mitochondrial ribosome [171]. Furthermore, Ssc1 interacts with different J-proteins which confer substrate specificity [172], as well as Mge1, the major mitochondrial nucleotide exchange factor [173]. As its cytosolic counterpart, it is not only needed during physiological conditions but also during stress for refolding of misfolded proteins [174].

After release by Hsp70, some proteins need further assistants with folding and therefore interact with the chaperonin Hsp60. As cytosolic Hsp60, mtHsp60 forms a folding chamber and Hsp10 builds the lid [167, 175]. Some proteins, however, can be folded by Hsp60 in the absence of Hsp10 [176]. Upregulation of mtHsp60 transcription is a major indicator for induction of the mitochondrial unfolded protein response (UPR\textsuperscript{mt}) [177]. The importance of mitochondrial proteostasis is also underlined by the finding that deletion of mtHsp60, Hsp10 or Ssc1 in yeast are lethal and that mutations in mtHsp60 are associated with neurological disorders in patients [168].

Hsp78 is the mitochondrial disaggregase in yeast and a member of the ClpB/Hsp100 family [129]. It shows a high sequence homology to the cytosolic disaggregase Hsp104 and can even replace Hsp104 function in hsp104 deletion strains [178]. Hsp78 itself was reported to be required for restoring mitochondrial function after, rather than during, stress [179]. As its cytosolic counterpart, Hsp78 was also shown to act in a bi-chaperone system together with Ssc1 and Mge1 [180]. Besides the interaction with the Hsp70 chaperone system, Hsp78 also cooperates with the proteolysis system [166, 168].

**Mitochondrial proteases**

Mitochondria are surrounded by two membranes. Thus, proteins residing inside the organelle cannot be targeted and degraded by the cytosolic ubiquitin proteasome system. In order to maintain mitochondrial proteostasis, and thereby cellular fitness and survival, the organelle contains its own proteases to degrade unassembled or misfolded proteins. As many other chaperones and proteases, mitochondrial proteases belong to the AAA+ superfamily of proteins (see section ‘Molecular chaperones’) [167, 181]. Here, I will focus on the three main classes of proteases.

The soluble serine protease Lon (Pim1 in yeast) resides in the matrix and resembles most closely the UPS in the cytoplasm [181, 182]. Main targets of Lon are oxidatively damaged proteins [167, 183]. Lon lacks an intrinsic unfolding activity probably to restrict degradation to unfolded and damaged proteins. Lon cooperates with the Hsp70 chaperone system [167].

Another soluble matrix protease, ClpXP, is found in mammalian mitochondria but its function is not well characterized yet [168]. The protease ClpP cooperates with the hexameric chaperone ClpX which unfolds the substrate and ensures substrate specificity of ClpP [181]. ClpP was reported to play a role in the UPR\textsuperscript{mt} (see chapter ‘Interorganellar communication’). Surprising-
ly, only a homolog for ClpX is found in yeast mitochondria (Mcx1) but not for ClpP [184].

The IMM represents the protein richest membrane in the cell as it harbors the OXPHOS complexes, many different carriers as well as the import machinery [167, 181]. For cell survival it is critical to keep these systems intact, explaining the need for a protease system in the IMM. Besides oxidatively damaged proteins, unassembled OXPHOS subunits are major targets of these proteases. There are two main metalloproteases in the IMM, i-AAA and m-AAA, both members of the FtsH/AAA family. Both proteases assemble in hexameric ring structures forming a proteolytic chamber but expose their catalytic sites on opposite sides of the IMM allowing surveillance of proteostasis on both sides of the membrane [168, 181, 182, 185]. Having a rather degenerate substrate specificity, they recognize membrane topology and folding state of proteins and 10 to 20 amino acids of solvent exposed stretches are sufficient for recognition and complete degradation of substrates [186].

In yeast, the m-AAA protease consists of alternating subunits of Yta10 and Yta12 and the catalytic site faces the matrix [187]. Not many endogenous substrates of these proteases are known yet and their proteolytic activity was mainly studied on imported model substrates. However, it was shown that unassembled OXPHOS subunits are substrates of the m-AAA protease [188, 189]. Besides complete degradation of proteins, the m-AAA protease was also shown to have non-proteolytic functions [190]. The prohibitins Phb1 and 2 are negative regulators of m-AAA protease. They form large assemblies in the IMM and interact with m-AAA protease [191]. The exact molecular basis of inhibition of m-AAA activity by Phb1/2 is not understood but it was shown that deletion of PHB1 increases degradation of unassembled complex IV subunits [192]. It was suggested that prohibitins might interact with and stabilize newly synthesized OXPHOS subunits to protect them from degradation [193]. Another possibility is that they affect protease activity by changing the lipid environment, as prohibitins have a scaffolding function and can compartmentalize the membrane [191].

The i-AAA protease is built by six Yme1 subunits and the catalytic domains face the IMS [194]. The substrates for i-AAA protease are still more elusive than for m-AAA protease, but unassembled Cox2 was shown to be a substrate [195, 196]. In contrast to the negative regulation of m-AAA protease by prohibitins, i-AAA protease is positively regulated by Mgr1 and Mgr3. Mgr1 and 3 form a complex and interact with substrates as well as with i-AAA protease, serving as an adaptor between them. Deletion of either protein decreases protein turnover by i-AAA protease [197, 198]. In addition to their proteolytic activities both proteases also have chaperone functions and play a role in OXPHOS assembly [167].
**Figure 10: Mitochondrial proteostasis network.** Mitochondria harbor a full set of chaperones as well as proteases to maintain organellar proteostasis. Hsp70 interacts with imported, newly synthesized as well as misfolded proteins to assist in folding. Hsp60, in concert with Hsp10, acts downstream of Hsp70. Hsp78 is the mitochondrial disaggregate and cooperates with the Hsp70 chaperone system. The Lon protease degrades soluble proteins in the matrix. The i- and m-AAA proteases are membrane anchored proteases (NEF, nucleotide exchange factor; IMM, inner mitochondrial membrane; IMS, intermembrane space; TIM, translocase of inner membrane).

**Sequestration compartments**

A recent study reported a new sequestration compartment called intramitochondrial quality control compartment (IMiQ) [199]. It forms upon heat stress as well as import of aggregation-prone model proteins into mitochondria and, as the name suggests, is located inside mitochondria. However, it is sequestered to the end of the mitochondrial network and colocalizes with mitochondria devoid of membrane potential. In contrast to cytosolic INQ which disappears a few hours after the stress release [142], a clearance of IMiQ was not observed within 24 hours. Further studies are needed to determine the contents of this aggregation site and follow its fate which might be clearance by mitophagy.
Interorganellar communication

Mitochondria are deeply integrated into the cells energy metabolism and signaling events. Furthermore, the central component of energy production, the OXPHOS system, is encoded by two separate genomes and expression needs to be coordinated. Having this in mind, it is not surprising that mitochondria constantly sent and receive signals from the nucleus as well as from the cytosol.

Mitochondria - nucleus communication

**Anterograde signaling**

Mitochondrial metabolism has to be adapted to cellular needs. Therefore, mitochondrial biogenesis needs to be tightly regulated and coordinated through changes in nuclear gene expression. The pathways involved are the main nutrient sensitive signaling pathways PKA, TOR and AMPK [110, 200]. Under conditions of high glucose, representing fermentative growth conditions in yeast, PKA and TOR are activated and repress mitochondrial biogenesis and OXPHOS components on a transcriptional level [151, 201]. In the presence of non-fermentable carbon sources as glycerol PKA and TOR are inhibited, allowing for mitochondrial biogenesis via activation of the transcription factors Msn2/4 [200]. As described in the section ‘Protein quality control in the cytosol’, PKA and TOR also respond to stress, resulting in a coordination of aerobe metabolism and stress signaling as shown by the similarities in gene expression in stressed and diauxic shift cells [200].

**Retrograde signaling**

*The retrograde response in yeast*

The retrograde response (RTG) describes induction of a transcription program in the nucleus induced by mitochondrial dysfunction in yeast [202]. Rtg1 and 3 are basic helix-loop-helix transcription factors which form a heterodimer and bind to R-box elements in the promoter of target genes [203, 204]. Partial dephosphorylation of Rtg3, which is mediated by Rtg2 promotes translocation of the heterodimer Rtg1/3 to the nucleus and its binding to target genes (Figure 11) [205]. The kinase Mks1, in contrast, phosphorylates Rtg3 thereby preventing its nuclear localization [206, 207]. Over 400 genes are regulated by these transcription factors including genes of the TCA cycle, mitochondria and peroxisome biogenesis as well as redox defense, ultimately leading to a remodeling of metabolism [202, 208]. Such a remodeling is not only activated by dysfunctional mitochondria when energy supply is low, but also during the diauxic shift, where the cells switch from fermentation to respiration. The RTG is interconnected with multiple other
signaling pathways, one of them being TOR signaling [200]. Reflecting a state of high energy, active TOR signaling inhibits Rtg1/3 gene expression [209, 210].

Even though the signaling sequence of the RTG is well studied, the signal which activates it remains more elusive. Originally the RTG was described in cells lacking mtDNA, but today also defects in OXPHOS assembly, mitochondrial proteostasis or mitochondrial energy metabolism are known to induce RTG signaling [202]. Likely a drop in ATP levels [211] or a drop in mitochondrial membrane potential [212], both resulting from mitochondrial dysfunction, are the signals.

Most mitochondria-to-nucleus signaling pathways were reported to influence lifespan in various model organisms. Along these lines, it was shown that activation of RTG signaling increases RLS [213]. Although there are no homologs for the Rtg proteins in metazoans, there are similar pathways which enable a signaling from mitochondria to the nucleus regulated by calcium concentration, ROS levels and ATP concentration [200, 214].

![Figure 11: Retrograde response in yeast. Dysfunctional mitochondria activate RTG signaling. Likely a drop in ATP concentration or mitochondrial membrane potential activates the transcription factors Rtg1 and 3 to translocate to the nucleus where they activate transcription of target gene (TCA cycle, tricarboxylic acid cycle; mt biogenesis, mitochondrial biogenesis) (figure modified from [110]).]

The mitochondrial unfolded protein response in C. elegans and mammals

Because maintaining mitochondrial proteostasis is crucial for cell survival, mitochondria contain their own set of chaperones and proteases to ensure proteostasis [167] as already described in the chapter ‘Mitochondrial protein quality control’. However, if the stress is too severe and the mitochondrial protein quality control system becomes overwhelmed, an adaptive response is activated. Such stress responses can be counted as a branch of protein quality control and they were described for bacteria, the cytosol (HSR) and for the endoplasmic reticulum (UPR<sub>ER</sub>) of eukaryotes.

The first description of a mitochondrial unfolded protein response (UPR<sub>mt</sub>) was already over 20 years ago [215]. Martinus et al observed an upregulation of mitochondrial hsp60 and hsp10 in rho<sup>0</sup> hepatozytes, which was distinctive from the general HSR. Today, the UPR<sub>mt</sub> is best studied in C. ele-
Accumulation of misfolded proteins inside mitochondria activates the protease ClpP which degrades these proteins [217]. The generated peptides are then exported by the peptide transporter HAF-1 [218] resulting in relocation of the transcription factor ATFS-1 from the cytosol to the nucleus. In detail, ATFS-1 contains a mitochondrial targeting signal (MTS) as well as a nuclear localization signal (NLS). Under non-stress conditions ATFS-1 is imported to mitochondria and degraded by the protease Lon. During mitochondrial stress, however, ATFS-1 is imported to the nucleus where it, together with the transcription factors Dve1 and Ubl5, induces expression of target genes including genes of mitochondrial biogenesis, antioxidative defense as well as mitochondrial proteases and chaperones [177, 218]. How do peptides activate translocation of ATFS-1 to the nucleus? Might it rather be a general inhibition of mitochondrial protein import by a decrease in membrane potential that causes the nuclear localization of ATFS-1? This statement is supported by the observation that HAF-1 is not vital for activation of UPR\textsuperscript{mt} [216]. On the other hand, also in yeast the release of peptides from mitochondria was suggested to play a role in mitochondria to nucleus signaling [219].

**Figure 12: Mitochondrial unfolded protein response in *C. elegans*.** Misfolded proteins inside mitochondria are degraded by the protease ClpP. The generated peptides are exported to the cytosol via HAF-1. By a yet unknown mechanism the peptides activate translocation of the transcription factor ATFS-1 into the nucleus where it activates transcription of target genes to restore mitochondrial proteostasis. In a second branch of the UPR\textsuperscript{mt} ROS released from mitochondria inhibit cytosolic translation via activation of the kinase Gcn2 and subsequent phosphorylation of the elongation factor 2α (ROS, reactive oxygen species; OXPHOS, oxidative phosphorylation; MTS, mitochondrial targeting signal; NLS, nuclear localization signal) (figure modified from [110]).
To lower the burden on the PN, downregulation of general cytosolic translation is a common response to stress. Also the UPR\textsuperscript{mt} results in a reduction of cytosolic protein synthesis. Gcn2, which is activated by an increase in ROS, phosphorylates the eukaryotic initiation factor 2\(\alpha\) (eIF2\(\alpha\)) [220]. P-eIF2\(\alpha\) inhibits general cytosolic translation but promotes translation of stress-induced proteins [221].

In mammals, the picture is not as clear as in C. elegans. ClpP degrades misfolded proteins inside mitochondria which are exported by a yet unknown mechanism, resulting in activation of the kinase JNK and subsequent phosphorylation of the transcription factor cJun. cJun then induces expression of CHOP and C/EBP\(\beta\) [222-224], which are two leucine zipper transcription factors involved in induction of gene expression of mitochondrial chaperones and proteases in response to mitochondrial stress [222, 225]. Similar to the response in worm, a reduction in cytosolic translation was reported [226].

The mammalian transcription factor ATF5 is likely a homolog of ATFS-1, as it can replace ATFS-1 function in worms. As ATFS-1, ATF5, is a leucine zipper transcription factor containing a MTS as well as a NLS and is involved in protective gene expression during mitochondrial stress [227]. However, it remains to be studied in the future if ATF5 localization is regulated by peptide export and how CHOP, C/EBP\(\beta\) and ATF5 play together.

More recently another protein, GPS2, with dual targeting sequence was reported to mediate mitochondrial retrograde signaling in mouse adipocytes during mitochondrial depolarization [228]. Taken the dual localization of ATFS-1, ATF5 and GPS2 together this might be a common mechanism of regulation of gene expression in response to mitochondrial stress.

Different perturbations inside mitochondria were shown to activate the UPR\textsuperscript{mt}. Besides mtDNA depletion, impaired mitochondrial protein quality control and defects in OXPHOS [177, 229], also changes in mitochondrial translation were reported to change nuclear gene expression in model organisms from yeast to mice [110].

**Stress signaling evoked by dysfunctional mitochondrial translation**

Several studies in yeast reported that deletion of certain mitochondrial ribosomal proteins activated retrograde signaling resulting in a changed nuclear gene expression and an increase in RLS [219, 230-232]. The exact nature of this response, however, seems to depend on the gene deleted and is not caused by a general defect in mitochondrial translation. This is also supported by the fact that deletion of mitochondrial ribosomal proteins in general did not induce such a response or change lifespan [230-232]. The following examples show how diverse the signals are when different components of the mitochondrial translation machinery are deleted.

Deletion of Sov1, the translational activator of the ribosomal protein Var1, increased RLS. This increase in RLS was dependent on the transcription
factors Msn2/4, which were activated by decreased PKA signaling, and on the deacetylase Sir2 [230].

An increase in RLS was also observed when the ribosomal protein Mrpl32 was deleted or its assembly into the ribosome was blocked. The increase in RLS was dependent on the transcription factor Gcn4 [231, 233]. In contrast, deletion of another ribosomal protein of the large subunit, Mrpl25, increased RLS through activation of Tor signaling. Activated Tor signaling resulted in nuclear localization of the transcription factor Sfp1 [232].

The increase in lifespan caused by deletion of mitochondrial ribosomal proteins was not only observed in yeast but is conserved in higher eukaryotes. In an RNAi screen, 22 mitochondrial ribosomal proteins or translation factors were identified to activate UPR\textsuperscript{mt} and change lifespan in \textit{C. elegans} [234]. In another study, knock down of Mrps5 in \textit{C. elegans} and mice increased lifespan by specifically activating the UPR\textsuperscript{mt}, but not UPR\textsuperscript{ER} or HSR. In contrast to the observations in yeast, this was accompanied by a decrease in mitochondrial translation causing a mitonuclear protein imbalance of OXPHOS subunits. Also inhibition of mitochondrial translation with doxycycline activated the UPR\textsuperscript{mt}. In line with this, deletions of other mitochondrial ribosomal proteins increased lifespan, pointing towards a more general mechanism [235]. This is different to the observations in yeast, where only deletion of certain mitochondrial ribosomal proteins increased lifespan but not others [230-232].

Mitochondrial stress and cytoplasmic proteostasis

Besides retrograde signaling changing nuclear gene expression in response to mitochondrial stress, more and more evidence accumulates for a mitochondria-cytosol communication axis. Specifically work in yeast has identified pathways where mitochondrial function impacts on cytoplasmic proteostasis [200, 214, 236-240].

In two studies published back to back in 2015 cytosolic accumulation of mitochondrial precursor proteins was shown to induce a response in the cytosol (Figure 13). By simultaneously decreasing cytosolic translation and increasing protein degradation, this response reduces the burden on the PN [236, 237]. In the first study, Chen \textit{et al} screened for multisuppressor genes of cell death induced by mitochondrial precursor overaccumulation stress (mPOS). Surprisingly, the suppressor genes identified were not mitochondrial proteins but instead involved in cytosolic proteostasis. Amongst others, the identified proteins are known to reduce cytosolic protein synthesis and induce protein degradation [236]. The identification of proteasome assembly factors was especially interesting as they were also found in the second study to be induced during mPOS. In that study, Wrobel \textit{et al} used a mutant which is impaired in mitochondrial protein import into the IMS to study the effects of mistargeted proteins (which activate mPOS) on cell physiology. An in-
crease in proteasome activity via proteasome assembly was observed, concomitant with a reduction in protein translation in the cytosol [237]. It was suggested that this UPR activated by mistargeted proteins (UPRam) is a protective response triggered by mPOS to lower the load on the PN and restore cytosolic proteostasis during import stress. Both studies show a previously undervalued communication between mitochondrial function and cytosolic proteostasis.

In line with these studies, IMS stress in mammalian cells also activated the proteasome [241] and was linked to longevity [242, 243]. The observed increase in proteasome activity caused by mPOS and IMS stress, however, stands in contrast to the previously shown proteasome disassembly caused by an increase in ROS during mitochondrial dysfunction [244, 245]. The opposite effects on the proteasome might stem from the different signals released from mitochondria (ROS versus mPOS).

A more recent study reported on another stress response activated by mPOS. The mitochondrial comprised protein import response (mitoCPR) surveils mitochondrial protein import by targeting proteins accumulating in the translocase channel or at the surface of mitochondria for degradation [238].

**Figure 13: Responses caused by mitochondrial dysfunction.** Cytosolic accumulation of mitochondrial precursor proteins due to dysfunctional mitochondria disturbs cytosolic proteostasis. UPRam reduces the burden on the PN by simultaneously decreasing translation and increasing protein degradation. mitoCPR also increases protein degradation. In parallel adaptive transcription programs are turned on by retrograde signaling (mPOS, mitochondrial precursor overaccumulation stress; UPRam, UPR activated by mistargeted proteins; mitoCPR, mitochondrial comprised protein import response; TOM, translocase of outer membrane; TIM, translocase of inner membrane).
In addition, an active role of mitochondria in clearance of aggregated proteins was proposed. The import of aggregated proteins from the cytosol into mitochondria was reported, where the proteins are degraded by the Lon protease Pim1. This mitochondria as guardian in the cytosol (MAGIC) response required the disaggregase Hsp104 as well as active mitochondrial protein import [246]. Though it remains open how cytosolic proteins should be imported by the highly selective mitochondrial import machinery in the absence of a MTS.

A boost in the research field of mitochondrial signaling and its connection to homeostasis was observed in 1996 after the discovery that apoptosis is triggered by the release of cytochrome c from mitochondria [247]. After 20 years of research in this field, many important findings have been made and it is well established that mitochondrial dysfunction activates protective responses to restore mitochondrial (retrograde signaling, UPR$^{mt}$) as well as cytosolic proteostasis (UPR$^{cyt}$, mitoCPR). Besides a well-defined RTG signaling in yeast and UPR$^{mt}$ in C. elegans, other signaling pathways remain more elusive and clearly need further investigation.
Aims

Mitochondria harbor the OXPHOS system which produces most of the cellular energy in form of ATP. The OXPHOS system is encoded by two genomes residing in different organelles. Consequently, to supply the cell with ATP, mitochondrial and nuclear gene expression need to be coordinated to allow for proper OXPHOS assembly and eventually ATP production. In contrast to bacterial translation, where all the steps are mechanistically and kinetically well characterized, mitochondrial translation is a process which is not yet defined on a molecular level. This lacking behind is mainly due to a missing in vitro translation system. Also the accuracy of mitochondrial translation remains elusive, as directed modification and, therefore, the use of mitochondrial encoded reporter proteins is not possible in mammals. Even though this is possible in yeast, it is not straight forward.

The work presented in this thesis focuses on mitochondrial translation in the model organism *S. cerevisiae*. Establishing methods to study timing, coordination and accuracy of mitochondrial translation was a major goal. Since the cryoEM structure of the mitochondrial ribosome from yeast as well as from mammals was solved, it is clear that the mitochondrial ribosome changed during evolution compared to its bacterial ancestor. However, the decoding center is highly conserved. Therefore, mutations in this region of the ribosome, which were shown to change bacterial translation accuracy, were used to study mitochondrial translation accuracy and its effect on cellular homeostasis and aging.
Summaries of papers

**Paper I – A novel system to monitor mitochondrial translation in yeast**

Mitochondria arose from the engulfment of an α-proteobacterium by an ancestral eukaryotic cell in a process called endosymbiosis. Even though, most of the mitochondrial genome was transferred to the nucleus during evolution, mitochondria still contain a small organellar genome. In yeast, this genome encodes eight proteins. To date, studying mitochondrial translation *in vivo* requires inhibition of cytosolic translation with the antibiotic cycloheximide. By inhibiting translation, however, levels of free amino acids increase, which activates TOR signaling and eventually changes cell growth, nutrient signaling and life span. Not only protein synthesis but also protein degradation is affected by cycloheximide and even a direct effect on mitochondrial translation was reported.

We established a mitochondrial encoded superfold GFP (GFP\textsuperscript{m}) which can be used as a reporter to follow mitochondrial protein synthesis. Using biolistic transformation we modified the mitochondrial genome in a way that GFP\textsuperscript{m} is expressed as a ninth protein and does not interfere with mitochondrial respiration. Using superfold GFP we aimed at improving the folding as it has faster folding kinetics and is more stable compared to normal GFP. We show that expression of GFP\textsuperscript{m} is regulated in the same way as of other mitochondrial encoded proteins and that expression can be followed via western blot, flow cytometry as well as fluorescent microscopy. Furthermore, after inhibition of mitochondrial translation or during glucose repression of mitochondrial biogenesis, the GFP\textsuperscript{m} signal decreased, making it a good reporter to detect changes in mitochondrial translation. With this system mitochondrial translation can be studied *in vivo* by visualizing GFP fluorescence without interfering with cellular homeostasis. This can be a pivotal tool to study timing, coordination and regulation of mitochondrial translation.
Paper II - Different genetic approaches to mutate the mitochondrial ribosomal protein S12

Over the last decades, an ever-growing number of methods and tools to modify the genome of the model organism *S. cerevisiae* became available. When the genome of yeast was first sequenced in 1996 in an international project, it became clear that many genes are conserved between humans and yeast. Therefore, and for many other reasons, yeast is a great eukaryotic model organism for basic research.

The most common method to study a protein and its implications in a certain pathway or process is deleting the gene by replacing it with a selection cassette using the yeast’s own homologous recombination system. The selection cassette can confer resistance to an antibiotic. Consequently, growth in the presence of this antibiotic can be used as selection to screen for successful recombination events. Alternatively, selection cassettes can be auxotrophic markers providing the cell with the ability to grow in the absence of a certain amino acid.

A mutated version of the protein can then be expressed from a plasmid or from the genome. Even though expression from a plasmid is standardly used, in some cases it is necessary to express the mutated protein from the genome.

Here, we aimed at using the *URA3* cassette, a counter selectable auxotrophic marker, to introduce a mutated version of the mitochondrial ribosomal protein S12 (Mrps12) into the genome of yeast. *URA3* encodes for Orotidine-5’-phosphate (OMP) decarboxylase, an enzyme required for uracil biosynthesis. Therefore, growth in the absence of uracil can be used as a first selection step. In the following step the *URA3* cassette is replaced by the mutated gene using 5-fluoro-orotic acid (5-FOA) for selection. 5-FOA is only toxic for cells still harboring the *URA3* cassette as it will be metabolized to the toxic compound 5-fluorouracil. In our hands, this method only gave false-positive results in the second selection step, most likely caused by loss of mtDNA during the first selection step. Loss of mtDNA is frequently observed when deleting mitochondrial ribosomal proteins and can cause a nuclear genome instability. This would explain why cells still harboring the *URA3* cassette, but a mutated version, can grow in the presence of 5-FOA. Moving forward with the deletion strain, we used an integrative plasmid instead to express the mutated *MRPS12* from the genome. After repopulating the cells with mtDNA, these mutants can be used to study translation accuracy in mitochondria as described in paper III.
Paper III – Mitochondrial translation efficiency controls cytoplasmic protein homeostasis

Mitochondria are mostly known for their role in production of the cells energy in form of ATP. Besides this crucial role, they also fulfill many other important tasks like harboring many metabolic processes, regulating calcium homeostasis and cell death. Disturbances in mitochondrial function are implicated in aging and disease. Therefore, it is not surprising that mitochondria are no isolated organelles but are in a constant crosstalk with other organelles to receive and sent signals.

Due to their endosymbiotic origin, mitochondria still contain a small organellar genome and the associated gene expression machinery. While the mitochondrial ribosome changed remarkably during evolution compared to its bacterial ancestor, the decoding site remained highly conserved. The decoding step during elongation determines the accuracy of the protein and is a trade-off between speed of peptide bond formation and accuracy of aminoacyl-tRNA selection. In bacteria, this step involves the ribosomal protein S12. Mutations in this protein were shown to change translation accuracy and were characterized by their resistance or hypersensitivity towards aminoglycosides. Aminoglycosides are a class of antibiotics binding to the small subunit of the ribosome and interfering with the decoding step by accelerating peptide bond formation and thereby decreasing translation accuracy. Consequently, error-prone S12 mutants were hypersensitive towards aminoglycosides while hyperaccurate strains were resistant.

Here, we introduced mutations in Mrps12, the mitochondrial orthologue of S12 in yeast. The mutations recapitulate the bacterial phenotype in regard to sensitivity towards aminoglycosides, the hyperaccurate mutant being resistant and the error-prone mutant being hypersensitive, indicating a conserved role in the decoding step. Furthermore, the error-prone mutant showed a shortened CLS, an increase in ROS levels during aging and a failure in handling cytosolic aggregates. During stress, it activated an interorganellar proteostasis transcription program (IPTP) upregulating mitochondrial biogenesis, proteases and chaperones besides the general heat shock response. Activation of IPTP was dependent on the stress transcription factors Msn2/4 downstream of TOR. In contrast, the hyperaccurate mutant repressed IPTP and an inappropriate activation was even disadvantageous. In addition, the hyperaccurate mutant showed increased CLS and increased proteostatic capacity in the cytosol. Together, these results demonstrate a direct crosstalk between mitochondrial translation output and nuclear gene expression as well as cytosolic proteostasis.
Paper IV - Insertion defects of mitochondrially encoded proteins burden the mitochondrial quality control system

Mitochondria are the powerhouse of the cell and produce most of the cells ATP via the OXPHOS system. Some subunits of the OXPHOS system are encoded in the mitochondrial genome, translated on mitochondrial ribosomes and co-translationally inserted into the IMM. Mitochondrial ribosomes are membrane anchored by different proteins, including Mrx15 and Mba1. Correct membrane insertion and assembly of mitochondrial translation products is pivotal for cellular ATP production. To ensure proper biogenesis and assembly of OXPHOS subunits, mitochondria also contain a whole set of chaperones and proteases building the mitochondrial proteostasis network. There are two IMM proteases, i-AAA and m-AAA, responsible for degradation of IMM proteins including unassembled or misfolded OXPHOS subunits. The i-AAA protease is regulated by Mgr1 and Mgr3, while the m-AAA protease is under control of Phb1 and Phb2.

Here, we showed that deletion of Mba1 increased sensitivity towards accumulation of mitochondrial misfolded proteins, while deletion of Mrx15 rendered the cells resistant towards mitochondrial proteotoxic stress. This suggests interaction of the ribosome receptors Mba1 and Mrx15 with components of the mitochondrial proteostasis network. Indeed, Mba1 showed genetic interaction with regulators of both IMM proteases. In contrast, Mrx15 only showed genetic interaction with the i-AAA protease regulators Mgr1 and Mgr3. In summary, these results are a first indication for a very early protein quality control step during OXPHOS biogenesis.
Future perspectives

How accurate is mitochondrial translation?
In this work I established a mitochondrial encoded reporter to follow orga-
nellar translation in respiring cells under physiological conditions (paper I).
Timing, regulation and coordination of translation can be studied with this
reporter but not accuracy. Therefore, it is a major task for future work to
establish a mitochondrial encoded reporter resembling the one used to mea-
sure error frequency in bacteria. There, a dual reporter construct consisting of
two reporter proteins connected by a short linker region is used to study
translation accuracy [248, 249]. Following expression of the first protein
serves as an internal standard. By introducing mutations in the linker region
or in the active site of the second protein error frequency can be measured
(Figure 14). An example for such a mutation would be a stop codon in the
linker region. If translation is accurate only the first protein is expressed. If
translation, however, is inaccurate both proteins are expressed due to a
readthrough of the stop codon. By calculating the ratio between the expres-
sion levels of the two proteins a number for error frequency can be estab-
lished.

Figure 14: Dual reporter system. A reporter consisting of two proteins connected
by a short linker region can be used to measure translation accuracy by calculating
the ratio between expression of both proteins. By introducing mutations in the linker
region readthrough events can be monitored.
Additionally, error frequency of bacterial translation is measured using an *in vitro* translation system where the exact incorporation of non-cognate or near-cognate tRNAs over cognate tRNAs can be followed. Having a similar system for mitochondrial translation would not only enable us to determine accuracy of translation but open up a whole new research field gaining mechanistic as well as kinetic insights into the single steps of mitochondrial translation.

We set out to get first insights into the role of mitochondrial translation accuracy for cell survival by relaying the knowledge gained from decoding in bacteria to mitochondria. Therefore, we introduced mutations into the highly conserved decoding center of the mitochondrial ribosome (*paper II*). Although we have good evidence suggesting a changed translation accuracy in our mutants based on their resistance and hypersensitivity towards the aminoglycoside paromomycin (*paper III*), this is only an indirect measure. Being able to determine translation accuracy in the mutants, either with a dual reporter system or an *in vitro* translation system, would be direct proof. Furthermore, albeit all our efforts to get estimates about the speed of translation in the mutants we were not able to detect any differences. In bacteria, it was shown that translation accuracy is a trade-off between speed of peptide bond formation and accuracy of aminoacyl-tRNA selection [250] and evolution most likely selected for the right balance between these two factors. In analogy, the hyperaccurate mutant should have a slower translation and the error-prone mutant should have a faster translation compared to wild type. Therefore, it would be interesting to be able to use an *in vitro* translation system to measure the kinetics of amino acid incorporation in the mutants.

**What is the signal released from mitochondria?**

During stress in form of nutrient deprivation, we observed activation of the protective IPTP in the wild type and error-prone mutant, which was repressed in the hyperaccurate mutant. This response was dependent on the transcription factors Msn2/4 (*paper III*). However, it remained elusive how changes in mitochondrial translation activate Msn2/4-dependent gene expression. Is it the drop in ATP due to nutrient deprivation which activates the stress program? This is unlikely as all three strains should experience the same drop in ATP levels but still show different responses. The error-prone mutant showed the strongest activation of IPTP and it could be argued that this is due to the decreased respiratory capacity of this mutant. On the other hand, also the hyperaccurate mutant showed a respiratory deficient phenotype but did not activate IPTP. For now it will remain an open question how changes in mitochondrial translation affects nuclear gene expression in our strains.

Furthermore, we showed that mitochondrial translation impacts on cytosolic homeostasis (*paper III*). However, the way of communication remained undiscovered. This is not the first time a crosstalk between the two orga-
nelles was observed and different signals were described to be part of this communication. It was reported that accumulation of mitochondrial precursor proteins in the cytosol would increase proteasome activity via activation of UPRam [237]. Yet, we did neither observe an accumulation of precursor proteins in the cytosol nor an increase in proteasome activity which would argue against an activation of UPRam in our mutants. However, we cannot exclude small changes in import kinetics from our experiments. Therefore, we would need to follow protein import into mitochondria or measure membrane potential which is crucial for protein import. Another obvious candidate would be ROS, especially because we observed changed sensitivity towards oxidative stress in our mutants. But even under strict anaerobic conditions we still observed a different proteostatic capacity excluding ROS as signaling molecule in our pathway. Furthermore, an additional copy of the mitochondrial superoxide dismutase (SOD2) could not prolong lifespan in the error-prone mutant. Being able to detect the signal sent from mitochondria to the cytosol will be a challenging task for future investigations.

**Is mitochondrial protein quality control happening at the ribosome?**

We showed a genetic interaction between proteins located at the tunnel exit of the mitochondrial ribosome which are responsible for membrane protein insertion (Mba1 and Mrx15) and regulators of the i-AAA and m-AAA proteases of the IMM (paper IV). This is first evidence that protein quality control might happen directly after protein synthesis to avoid accumulation of aggregation-prone hydrophobic proteins. A recent publication from our group also supports this idea, as both AAA proteases were copurified with the ribosome indicating colocalization [251]. To proof a direct involvement of Mba1 and Mrx15 in mitochondrial proteostasis, clearly further experiments are needed. A good starting point would be to monitor mitochondrial proteostasis in the absence of either of these proteins.

In bacteria, a similar scenario is observed where the homologs of m-AAA protease, Oxa1 and Phb1/Phb2 interact and form a complex. Here, degradation of misassembled membrane proteins is mediated by a chaperone function of YidC, the Oxa1 homolog, at a very early step during or directly after membrane insertion [252]. If Mba1and Mrx15 also form a complex with i-AAA protease, m-AAA protease and their regulators remains to be investigated.
Sammanfattning på svenska


Förutom rollen som cellens kraftverk är mitokondrier även inblandade i många viktiga signalprocesser som bestämmer cellens välmående. Det är därför inte överraskande att mitokondrier inte är isolerade organeller utan är istället i konstant kommunikation med andra delar av cellen.

I detta arbete använde jag bakjästen *Saccharomyces cerevisiae* som en modellorganism för att studera olika aspekter av proteinsyntes i mitokondrier. I den första artikeln skapade jag en reporter för att övervaka mitokondriell proteinsyntes. Denna reporter tillåter mer detaljerade studier av timing och reglering av den mitokondriella proteinsyntesen. I den andra och tredje artikeln använde jag tidigare kunskap om proteinsyntesen i bakterier, mitokondriernas förfäder, för att studera noggrannhet i mitokondriell proteinsyntes och dess konsekvenser för cellen. Vi visade att noggrannhet i mitokondriell proteinsyntes bestämmer cellens stresshantering och livslängd. I den sista artikeln fann vi några första bevis för en mycket tidig kvalitetskontroll under eller direkt efter den mitokondriella proteinsyntesen. Denna övervakningsmekanism kan undvika skadlig ackumulering av oveckade eller ej sammansatta proteiner såväl som felaktiga andningskedjekomplex, varigenom mitokondriell funktion kan säkerställas.
Many people contributed to the success of this thesis. First of all, Martin, you gave me the opportunity to do my PhD in your lab. I learned so much from you, especially to always keep a positive attitude and never give up, no matter how frustrating it is. You never doubted that it will pay out eventually – and it did!

I would like to thank my co-supervisor Elzbieta Glaser and my mentor Pia Ädelroth for their support.

Dina Petranovic and Thomas Nyström, you gave me the great opportunity to spent time in your labs in Gothenburg. You and your groups helped me significantly with my experiments.

Claes Andréasson, Sabrina Büttner, Per Ljungdahl, thank you for all the intense discussions during our Friday yeast meetings and beyond. I enjoyed getting new ideas from a “non-mitochondria-centered” point of view. Claes, without your help and great input my paper would not have become such a great story.

Thanks to all the past and present members of the Ott group. When I started my PhD we were a German group of six people, which changed dramatically over the last years to a group of 12 people from six different countries. Kirsten, without you I would not have survived the first year of my PhD. You cheered me up when something did not work out as planned (which happened constantly) and you shared your endless knowledge with me. Braulio, we started our PhD at the same time and it was fun sharing this incredible experience with you. Even if we had our doubts from time to time – we did it! Hannah, you were my first student and I am glad you decided to stay in our group. I will miss our coffee breaks during which we, of course, only discussed about science. Kathi, it was fun working on the bench next to you and chatting about scientific and non-scientific topics.

Povilas and Aziz, I enjoyed sharing an office with you for almost five years. We laughed so much together, making the days full of failed experiments bearable.
Anna and Nandu, I don’t know how many times I came over to your lab asking for help and you were never tired of sharing all your knowledge about chaperones with me. Since the paper is accepted, I sometimes catch myself looking for excuses to be able to stop by your lab.

Lukas, you made it possible that the GFP manuscript was submitted within no time. It was fun working with you and exchanging our knowledge about the best model organism of all.

Agata, I am deeply grateful for the bioinformatics support you gave us.

All the people who made the last six years in Sweden such a great experience. Hannah, Jacob, Braulio, Caro, Kirsten, Kathi, Jörg, I enjoyed all our get-togethers, especially the barbecues.

Last but not least, I could not have achieved all this without the constant support of my family. Papa, Andrea, Melissa und Romina, ich weiss ich kann immer auf euch zählen, was auch passiert. Dieses Wissen gibt mir die Sicherheit und den Mut meine Träume zu verfolgen. Mama, ich weiss, du bist stolz auf mich. Melissa, Romina und Hannes, eure vielen Besuche waren immer eine willkommene Abwechslung zu unserem Alltag.

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