PhD thesis

Analysis of two mitochondrial functions in *Saccharomyces cerevisiae*

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Introduction

Mitochondrion is an essential cellular organelle

To date, only one mitochondrial biosynthetic process, the assembly of iron–sulphur (Fe/S) cluster is known to render mitochondria essential. A long-standing question of mitochondriology therefore is why this proccess makes mitochondria, and thus Fe/S protein biogenesis, so important for life. Our goal was to answer this question in collaboration with a research group from Germany.

Our hypothesis was that mitochondria activate at least one essential Fe/S protein, the crucial function of which renders mitochondria indispensable for eukaryotes. Conspicuously, all the known essential Fe/S proteins are involved in the biosynthesis of Fe/S cluster.

After searching protein databases for proteins that are essential, contain at least one Fe/S cluster-binding sequence and have an unknown function, we chose Rli1p as a candidate protein for further studies. This protein is one of the most highly conserved proteins in evolution present in virtually all Eukarya and Archaea. At the beginning of our collaboration, we provided evidence that Rli1p binds Fe/S clusters but it is not involved in Fe/S protein biogenesis, therefore the maturation of the essential Fe/S protein Rli1p provides the first known process that explains the indispensable character of mitochondria for yeast cell viability.

Regulation of mitochondrial gene expression

In *Saccharomyces cerevisiae* most mitochondrial RNAs are produced by processing of polycistronic precursors that are transcribed from at least 13 distinct promoters, containing different combinations of rRNAs, tRNAs and mRNAs. The levels of cotranscribed mature RNAs can differ substantially, indicating that posttranslational mechanisms play an important role in governing transcript abundance. Despite their importance, we are just starting to gain a better understanding of mRNA degradation and its complex regulation in eukaryotic cells.

The mitochondial COB gene encoding for apocytochrome *b* is cotranscribed as a unit with the upstream $tRNA^{glu}$ gene. The mature mRNA is generated by cleavage of the 5'-end of the pre-mRNA at position –955 and –954. Cbp1p specifically helps the regulation of COB mRNA stability by binding directly to a sequence within the AU-rich COB mRNA 5' UTR that contains a unique CCG trinucleotide at position –942 to –944. Single-base changes in the CCG trinucleotide eliminate COB mRNA accumulation and reduce the level of precursor RNA 5-fold, a phenotype equivalent to that of *Δcbp1* mutants.

The proximity of the CCG trinucleotide to the 5'-end of the mature COB mRNA raised the hypothesis that maturation and degradation are connected: Cbp1 binding might protect the 5'-end of mRNA from degradation by nucleases.

Since the nuclease important in the maturation of COB mRNA has not been identified yet, this can be conceived in the following ways: Cbp1 binding to the CCG-containing region near the 5'-end of the mRNA may act as a roadblock to an exonuclease, or, alternatively, Cbp1p may cover an endonuclease cleavage site that is required for mRNA turnover.

Aims

In the first part of our work we explored the essential character of mitochondria by studying the function of Rli1 protein. Our objectives were:

- determining the subcellular localization of Rli1p,
- studying the role of protein structure in the essential character of Rli1p,
- identifying putative cellular interaction partners of Rli1p,
- analysis of the intracellular effects of Rli1p depletion.

In the second part we studied the mechanism of maturation and decay of the mitochondrial COB mRNA. Our aims were:

- constructing yeast strains with tandemly arranged functional and/or non-functional Cbp1 recognition sites,
- determining whether COB mRNA is processed at the 5' end from a longer precursor by an endonuclease or an exonuclease,
- investigating the importance of Pet127p in maturation and degradation.

Materials and Methods

Bacterial and yeast strains

The following *Saccharomyces cerevisiae* strains were used: **W303** (*MATα/a,ura3-1, ade2-1, trp1-1, his3-11,15, leu2-3,112*) in diploid and haploid form served as wild type. Disruption of the *RLI1* gene was achieved by PCR-based gene replacement technique yielding the diploid **RLI1/Δrli1** cells. Addition of a C-terminal TAP tag was achieved by adding the TAP tag coding sequence to *RLI1*. The resulting fusion gene was cloned into the pBS1479 vector and integrated into the yeast genome to yield **RLI1-TAP** strain. The **IRHA** strain was produced by integrating *RLI1* gene contained by the pYIplac211/HA integration vector into the yeast genome. Exchange of the endogenous promoter of the *RLI1* gene for a tetracycline-regulatable promoter to yield strain **Tet-RLI1** was performed by integrating the promoter substitution cassette of the pCM225 plasmid upstream of the *RLI1* gene. The PCR-amplified cassette was transformed into W303a haploid cells for homologous recombination.

High velocity microprojectile bombardment was used to construct strains with two tandemly arranged **Cbp1 recognition sites** within the mitochondrial *COB* gene. The *Pet127::KanMX4* fragment amplified by PCR was transformed into these yeast mitochondrial mutant strains for the generation of **Δpet127** strains. Yeast cells were grown in rich (YP) or minimal media (MM) containing the required carbon sources (2% glucose or 3% glycerol, respectively). 2% agar was added to these media to yield solid plates. For *E. coli* LB medium was used, the cultures were propagated at 37 **°C.**

Tetrad analysis

RLI1/Δrli1 cells grown on sporulation plates for 4-5 days were treated with 10% Glusulase. The suspension was pipetted onto a YPD plate and the spores were dissected using a micromanipulator. The growing haploid cells were observed after incubation at 30 °C for 5-6 days.

Differential centrifugation

IRHA cells were grown to mid-log phase in liquid YPD medium and were extracted using glass beads. The extract was subjected to a series of centrifugation steps with increasing velocity (3000 rpm 10 min, 10000 rpm 10 min, 100000 rpm 60 min) in order to separate different cellular organelles. Mitochondria were purified from the crude mitochondrial fraction by Nycodenz density gradient centrifugation. Proteins from different fractions were identified using Western blotting.

Sucrose density gradient centrifugation

RLI1-TAP cells grown in liquid YPD medium to OD_{600}=0.8 were extracted using glass beads. After centrifugation the supernatant was loaded onto a 10-50% linear sucrose gradient, then it was centrifuged with 40000 rpm for 5 hours at 4 °C using

Beckman SW41 rotor. Samples were collected using a fraction collector and analysed with Western blotting.

TAP-tag affinity purification

RLI1-TAP cells grown in liquid YPD medium to OD_{600}=0.8 were extracted using glass beads. IgG Sepharose bead suspension was added to the extract, then it was rotated for 90 min at 4 °C. The collected beads were treated with TEV protease and the cleaved proteins were separated from the beads by centrifugation. The supernatant containing the cleaved proteins was rotated with Calmodulin bead suspension for 60 min at 4 °C followed by washing of beads and subjecting them to Calmodulin elution buffer to elute the bound proteins.

Pulse-Chase Experiment

Wild-type and Tet-RLI1 cells were grown in liquid YPD medium in the presence or absence of doxycycline to logarithmic phase. Cells were collected, washed and re-suspended in minimal media lacking methionine and cysteine. Cells were incubated with 10 μCi ^{35}S methionine/cysteine for 10 min, following additional incubation for 10 min with 6 mM unlabeled methionine and cysteine. Collected cells were extracted and analysed using 12% % SDS-PAGE. Proteins containing the incorporated isotope were observed using PhosphoImager.

Analysis of respiratory competence

The strains were cultured in liquid YPD medium to OD_{600}=0.8. Cells were counted in a hemacytometer and serially diluted with distilled water to concentrations of 10^4, 10^3, 10^2, and 10 cells/6 μl. 6-6 μl drops of the serial dilutions were spotted onto glycerol plates and incubated at 30 °C for 12 days. The plates were photographed at days 2, 4 and 12 of incubation.

Primer Extension Analysis

Primers were end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. 8 μg of total cellular RNA and 10 pmol 5'-end-labeled primers were mixed in annealing buffer. The mixture was heated at 85 °C for 5 min and annealed at 45 °C for 90 min. Reaction mix containing dGTP, dCTP, dATP, dTTP and reverse transcriptase was added to the annealed primer-RNA mixture, then incubated at 42 °C for 45 min. Equal amounts of each reaction mixture were loaded on a 6% polyacrylamide-7 M urea sequencing gel. The results were visualized using PhosphoImager.

Results and Discussion

Investigation of the role of Rli1p: why are mitochondria essential?

The intracellular localization of Rli1p

To determine the subcellular localisation of Rli1p, cells expressing the HA-tagged version of Rli1p were fractionated by differential centrifugation, then the subcellular localization of our protein was tested by Western blotting. A smaller amount of Rli1p was present in the cytoplasm, whereas the remainder was recovered in the microsomal pellet fraction. To evaluate this result, our German partner group used *in situ* immunofluorescence microscopy to study the localisation of Rli1p. The majority of Rli1p was found to localize to the cytoplasm, while a weak fluorescence in the nucleus indicated that a small amount of Rli1p may reside in this compartment. The amount of Rli1p in the nucleus increased significantly when the nuclear export was inhibited.

Our data show that the majority of Rli1p resides in the cytosol, but a fraction shuttles between the nucleus and the cytosol.

The Fe/S clusters of Rli1p are essential for yeast cell viability

To confirm the essential character of Rli1p, tetrad analysis of a diploid RLI1/**Δrli1** yeast strain in which one *RLI1* allele was deleted by gene replacement through homologous recombination was used. After sporulation, the resulting spores were analysed for viability by tetrad dissection, which always gave rise to two viable spores confirming the indispensable role of this protein in yeast.

Each of the two [4Fe-4S] clusters of Rli1p binds to the apoprotein through four highly conserved cysteine residues found within its ferredoxin motif. To examine whether Fe/S clusters represent an essential part of Rli1p, one cysteine residue from the first, the second or both ferredoxin-like motifs were mutated to serine residues by PCR-based mutagenesis. Plasmids encoding Rli1p-HA with or without these mutations were transformed into diploid RLI1/Δrli1 cells. Cells harbouring plasmids encoding wild-type *RLI1* gave rise to four viable spores. Mutation of cysteine residue(s) at position 25 and/or 61 of Rli1p resulted in a 2:2 segregation of the spores, proving that Δrli1 cells harbouring mutant Rli1p were inviable.

These experiments demonstrate the crucial character of Fe/S clusters of Rli1p for viability of yeast cells.

ATP-binding of Rli1p is essential for yeast cell viability

To deplete Rli1p, we constructed the Tet-RLI1 yeast strain in which the *RLI1* gene was under the control of a tetracycline-repressible promoter. Addition of the tetracycline-related compound doxycycline to these cells led to a strong depletion of the RLI1 mRNA and Rli1 protein levels.

In the presence of doxycycline Tet-RLI1 cells containing the exogenous, wild-type *RLI1* gene grew similarly to wild-type cells, indicating that the exogenous Rli1 protein was able to substitute the depleted endogenous protein. Tet-RLI1 cells expressing Rli1p with ATP-binding lysine residues mutated to leucines within their first or second ATP-binding domains were unable to grow in the presence of doxycycline.

The lack of complementation shows that the ATP-binding ability of Rli1p is indispensable for the function of the protein, thus for the whole cell.

Rli1p is associated with Hcr1p *in vivo*

Recent studies have identified an interaction between Rli1p and Hcr1p, a protein involved in rRNA processing and translation initiation.We performed yeast two-hybrid analysis to study whether the Fe/S clusters and the nucleotide-binding domains of Rli1p were needed to establish this interaction.

Our results indicated strong interaction between Rli1p and Hcr1p *in vivo*.

Rli1p proteins with single mutation in one of their ferredoxin-like motifs were able to bind to Hcr1p, however, proteins with mutations in both motifs did not interact with Hcr1p. Neither Rli1 proteins mutated in their ATP-binding domains, nor the ATP-binding domain alone was able to bind to Hcr1p.

Our results indicate that the lack of one or the other Fe/S cluster resulted in a minor conformational change, that enables Hcr1p binding. However, upon the loss of both complexes, the structural change of Rli1p was so significant, that it prevented the interaction. Furthermore, the presence of the nucleotide-binding domain, hence ATP binding, proved to be crucial, although not sufficient for the interaction between Rli1p and Hcr1p.

Rli1p is associated with cytosolic ribosomes

For the identification of putative binding partners of Rli1p, we used Tandem Affinity Purification method. Numerous proteins of the large (60S) and small (40S) subunits of cytosolic ribosomes were identified as binding partners of Rli1p suggesting that our protein was associated with 80S cytosolic ribosomes and/or 60S and 40S ribosomal subunits.

To obtain an independent support for an interaction between Rli1p and cytosolic ribosomes, we fractionated cell extracts by sucrose density gradient centrifugation. The result confirmed that Rli1p bound to the 80S ribosomes and each ribosomal subunit.

Rli1p is required for protein translation

Binding of Rli1p to ribosomes implicates that this protein has a role in translation-associated processes. To address the potential role of Rli1p in protein synthesis, wild-type and Tet-RLI1 cells were treated with doxycycline in order to deplete Rli1p, they were briefly radiolabelled with ^{35}S-methionine and ^{35}S-cysteine to visualize the efficiency of protein synthesis.

Our result showed strongly impaired protein translation due to the depletion of Rli1p.

Rli1p is required for ribosome export from the nucleus

The analysis of the importance of Rli1p in the biogenesis of ribosomes was performed by our German partner lab. In order to follow the biogeneis of the ribosomal subunits, fusion proteins of the green fluorescent protein and one of the large and small ribosomal subunit proteins were expressed in wild-type and Tet-RLI1 cells. Mainly cytosolic localisation was seen in doxycycline-treated wild-type cells or in untreated Tet-RLI1 cells, whereas upon depletion of Rli1p, both ribosomal fusion proteins accumulated in the cell nucleus.

This clearly shows that Rli1p is necessary for ribosome biogenesis and/or nuclear export of both ribosomal subunits to the cytosol.

The Fe/S clusters of Rli1p are essential for ribosome biogenesis

We finally examined whether the lack of the Fe/S complex has an impact on the nuclear export of ribosomes, or the apoprotein alone is sufficient to fulfill its role in this process.

To answer this question, we used a mutant yeast strain defective in the assembly of cytosolic Fe/S proteins. The subcellular localisation of a GFP-tagged protein of the large ribosomal subunit was analyzed by our German partners using immunocytochemistry. When incorporation of Fe/S clusters into the Rli1p apoprotein was inhibited, strong nuclear accumulation of the large ribosomal subunit was seen, similarly to the effect observed during Rli1p depletion. Our data demonstrate that the presence of Fe/S clusters on Rli1p is crucial for its function in ribosome export from the nucleus.

Our results demonstrate a surprising link between the essential character of mitochondria for cell viability and the biogenesis and functionality of cytosolic ribosomes. Mitochondria, as central organelles for Fe/S protein biogenesis, play a decisive role in the generation of a functional protein synthesis machinery through the activation of Rli1p.

The investigation of COB mRNA: how the maturation and decay of mitochondrial mRNA from 5'-end takes place

We investigated the 5'-end decay of mitochondrial mRNAs by studying the turnover of COB mRNA. During the 5'-end maturation of COB pre-mRNA, depending on an exonuclease or endonuclease cleavage, the end-product can be remarkably different. In case of an exonuclease the 5' end is determined by the most 5' CCG element, while in case of an endonuclease the 5' end is determined by the position of the CCG element closest to the first coding nucleotide. We took advantage of this difference to detect the type of nuclease involved in the maturation of COB mRNA.

Construction of tandem Cbp1 recognition site

In addition to the *C strain*, containing the wild-type (CCG) Cbp1 recognition site, and the *A strain*, containing the mutant, nonfunctional (ACG) Cbp1 recognition site, the following strains were constructed:

- *AA strain*: contains two mutant (ACG) elements in tandem within the 5' UTR of the mitochondrial COB mRNA,
- *AC strain*: the upstream mutant (ACG) element was arranged in tandem with the downstream wild-type (CCG) element,
- *CA strain*: the upstream wild-type (CCG) element was arranged in tandem with the downstream mutant (ACG) element,
- *CC strain*: contains two wild-type (CCG) elements in tandem within the 5' UTR of the mitochondrial COB mRNA.

Both the upstream and downstream Cbp1 recognition sites are active

To determine whether the CCG element could function both upstream and downstream of the inserted sequences, respiratory phenotypes of strains containing a wild-type (CCG) and a mutant (ACG) element arranged in tandem (AC and CA strains) were compared with those of C and CC strains. Our observation was that both CC and CA strains grew very similarly to the wild-type C strain. The AC strain was also respiratory-competent, although it grew more slowly on glycerol.

This result verifies that the CCG site is operative in both the upstream and downstream positions.

COB pre-mRNA 5' UTR is processed by an exonuclease

The 5'-end of COB mRNA was mapped by primer extension analysis in strains containing different combinations of 64-nucleotide elements containing either the functional or the mutant trinucleotide. The 5' ends of both the mature and precursor COB mRNAs in the CC strain were identical to those in the CA strain, suggesting that only the upstream site defines the position of the 5' ends of the mRNA.

These data prove that the 5' end is determined by the most 5' functional Cbp1 recognition site, confirming that the maturation of COB pre-mRNA is due to the

action of an exonuclease that degrades the COB mRNA in the 5' to 3' direction until it is blocked by Cbp1p.

The exonuclease reaction is a Pet127p-dependent process

It is known that Pet127p has a role in the maturation of more mitochondrial RNAs including COB mRNA. To address the key role of the Pet127 protein in the degradation of COB mRNA, the nuclear *PET127* gene was deleted in all six strains with single and tandem 64-nucleotide elements in the 5' UTR of the COB mRNA. All these strains were respiratory-competent. The ability of these strains to grow on glycerol demonstrates that they have sufficient amount of COB mRNA to support respiration in the absence of Pet127p even when Cbp1p cannot bind to COB mRNA. The analysis of 5' ends of COB mRNAs in these cells determined by primer extension analysis demonstrated that the absence of Pet127p leads to an almost complete disappearance of mature COB mRNA and to the enrichment of the precursor form.

This result strongly supports the hypothesis that 5' processing and degradation of COB precursor mRNA by an exonuclease are linked processes that largely depend on the presence of Pet127p.

In our present study we proved that in *Saccharomyces cerevisiae*, the maturation and decay of mitochondrial COB mRNA encoding apocytochrome *b* is linked in a Pet127-dependent 5'-3' exonuclease reaction. This is the first 5'-3' exonucleolytic activity observed to date in mitochondria.

Summary

During our study of the indispensable character of mitochondria, we investigated the role of Rli1p, an essential yeast Fe/S protein with poorly defined function:

- We showed that the majority of Rli1p resides in the cytosol, but a smaller fraction shuttles between the nucleus and cytosol.
- We proved that Rli1p requires both Fe/S clusters and its ATP binding ability to perform its indispensable function.
- We reported a tight association of Rli1p with cytosolic ribosomes and Hcr1p, a protein involved in rRNA processing and translation initiation.
- We found that the depletion of Rli1p resulted in a strong defect in the nuclear export of the small and large ribosomal subunits to the cytosol and caused impairment of protein synthesis.
- Our data demonstrate that the presence of Fe/S clusters on Rli1p is crucial for its function in ribosome export from the nucleus.

In this work we provided *in vivo* evidence that Rli1p performs a crucial role in the biogenesis of ribosomes. As the assembly of the Fe/S clusters is strictly dependent on several components of the mitochondrial Fe/S protein assembly machinery, maturation of the essential Fe/S protein Rli1p provides the first known process that explains the indispensable character of mitochondria for yeast cell viability.

To investigate the regulation of mitochondrial gene expression we analysed the 5'-end maturation and decay of mitochondrial COB mRNA:

- We constructed strains with two Cbp1 recognition sites in tandem within the 5' UTR of COB mRNA with the wild-type (CCG) and/or the mutant (ACG) element.
- We proved that the processing and degradation of COB pre-mRNA are linked.
- We showed that these reactions are due to the action of a 5'-3' exonuclease.
- We demonstrated the key role of Pet127p in the regulation of COB mRNA turnover in the mitochondria.

This is the first mitochondrial RNA-decay system identified to date having 5'-3' directionality and 5'-3' exonuclease activity.