Author

During the summer after her freshman year, Reshmi discovered the opportunity for research that led her to work at UCI's Cumsky Laboratory. According to Reshmi, participating in research has enhanced her education in more than a few ways, and has given her a foundation for pursuing her goal of attending medical school. "I have been made aware of the limitless scope of research and the potential impact it can have on everyday life," she says. She is an active member of UCI's Flying Samaritans, enjoys playing the piano and collecting first edition books.

Key Terms

- Essential Gene
- *MIA1*
- Mitochondria
- Respiration
- *Saccharomyces cerevisiae*

Characterization of the Essential Gene *MIA1* in *Saccharomyces cerevisiae*

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Abstract

The biogenesis of functional mitochondria is essential to the survival of all eukaryotic cells. Mitochondria are organelles whose chief function is to supply a cell with energy in the form of adenosine triphosphate (ATP). The final step in ATP production requires cytochrome c oxidase, a multi-subunit enzyme complex. One of the essential subunits of cytochrome c oxidase is Va. Ongoing studies seek to identify the mitochondrial proteins responsible for the import and sorting of subunit Va to the mitochondrial inner membrane. Past research in the Cumsky laboratory has identified a previously uncharacterized gene named *MIA1*. This gene is encoded by nuclear DNA, encodes a 16.2 kDa gene product localized in the mitochondrial inner membrane, and is essential for the viability of yeast cells. The goal of this project was to investigate the role of the Mia1 protein (Mia1p) in mitochondrial biogenesis. It was found that cells depleted of Mia1p show loss of respiratory complexes, lose mitochondrial DNA, and display altered mitochondrial morphologies. *MIA1* has a human homolog whose product, CG1-136 protein, has 65% similarity in amino acid sequence. Therefore, it is probable that defects in the *MIA1* homolog in humans will result in severe disease. Further understanding of protein import and localization in mitochondria may help with the development of therapies that will address such problems.

Faculty Mentor

When Reshmi began work on the *MIA1* gene, our lab group knew little about it. Her work, and that of her primary collaborator Dr. Virginia Bilanchone, established that *MIA1* encodes a protein critical for mitochondrial biogenesis. Furthermore, this work determined the scope and direction of subsequent studies that have confirmed Reshmi's findings. These studies have shown that the *MIA1* protein interacts with proteins of the mitochondrial import motor, and therefore may be a component of this molecular machine. In addition, it was found that a Drosophila homolog of *MIA1* causes early embryogenic death in fruit flies when defective. Reshmi's project is a wonderful example of excellence in undergraduate research. It also underscores the value of this experience for both student and laboratory. Reshmi's work is a significant contribution to both our laboratory's research and mitochondrial research in general.

Michael Cumsky
School of Biological Sciences

Introduction

All eukaryotic cells require the biogenesis of functional mitochondria for viability. Mitochondria are organelles that operate in the metabolism of heme proteins and iron (Beinert et al., 1997) and provide much of the biochemical energy required to sustain normal biological functions. Adenosine triphosphate (ATP) stores this energy and is produced by the interaction of different proteins within the mitochondria. The final step in ATP production requires cytochrome c oxidase (CCO), a multi-subunit complex. In eukaryotes, CCO is a heteroligomer containing up to 13 different polypeptide subunits that are products of both the nuclear and mitochondrial genomes. The three largest subunits (I, II and III) are the products of mitochondrial DNA and are transcribed and translated within the organelle. The remaining subunits are nuclear gene products and are imported into the mitochondria after translation in the cytoplasm. One of the essential subunits of CCO, Va, contains the protein COX5a that is encoded by nuclear DNA and synthesized outside the mitochondria in the cytoplasm. Mitochondrial gene products of nuclear origin, such as COX5a, must be targeted to the mitochondria and then sorted to one of the four intra-mitochondrial compartments. These mitochondrial proteins must not only be imported from the cytoplasm, but they must also be properly localized within the organelle (Lill et al., 1996). Therefore, many genes involved in mitochondrial import and sorting have proven to be essential for the survival of eukaryotic cells (Voos et al., 1999).

Previous investigations in the Cumsky laboratory have utilized a high-copy DNA library to identify the components inside yeast mitochondria that are responsible for import and sorting of COX5a into the mitochondrial inner membrane. The approach is based on the phenotype of cells containing the mutant protein COX5a109R. The incorrect sorting or localization of this mutant protein results in a defect in cytochrome c oxidase assembly and loss of CCO activity (manuscript in preparation). The genetic screen selects for wild-type genes, which, when present in high copy, suppress the growth defect of cells containing the mutant protein. Through this screen, the Cumsky laboratory has thus far identified several genes involved in the import and/or assembly of CCO. One of these genes, *MIA1*, represents an open reading frame that has not previously been characterized. Through previous experiments it has been found that *MIA1* is encoded by nuclear DNA, localized in the mitochondrial inner membrane, and specifies a 16.2 kDa gene product that is essential for the survival of yeast cells (unpublished).

The current study sought to investigate the role of the protein encoded by *MIA1* in the cell. Because *MIA1* is essential, yeast cells will die without the gene product and so a system of conditional expression needed to be established. This was accomplished by constructing a plasmid, pgal*MIA1*, in which the native promoter of *MIA1* was replaced with the *GAL1* promoter. Expression of genes positioned adjacent to this promoter can be controlled at the level of transcription and induced or repressed by growing cells in media containing galactose and glucose respectively.

This system of conditional expression was used to examine the effects of Mia1 protein (Mia1p) depletion in *Saccharomyces cerevisiae* cells. The results indicate that cells depleted of Mia1p lose CCO activity, lose respiratory proteins and complexes, display altered mitochondrial morphology, and lose mitochondrial DNA. These findings are consistent with the hypothesis that Mia1p is involved in the biogenesis of respiratory complexes in mitochondria.

Materials and Methods

Yeast Strains and Media

The following *S. cerevisiae* stains were used: W303.1a (*MAT*a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1*) (R. Rothstein); *Dmia1* (*MAT*a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 Dmia1*:: LEU2), a derivative of W303.1a, in which the chromosomal *MIA1* gene was replaced with the yeast *LEU2* gene (D. Lalo). Cells were grown in YPGal (yeast extract, bacto peptone, 2% galactose) or YPD (yeast extract, bacto peptone, and 2% dextrose).

Plasmid Construction

The plasmid p*MIA1* was constructed by inserting the 1349 base-pair (bp) BamHI-SnaBI *MIA1* fragment into the BamHI-SmaI sites of pRS314 (D. Lalo). The plasmid pgal*MIA1* was constructed by replacing the native promoter of *MIA1* in p*MIA1* by the *GAL1* promoter at a position 4 bp upstream of the translation start site.

Growth Curve Assay Conditions

The *Dmia1* cells containing either p*MIA1* or pgal*MIA1* were grown in YPGal media at 30 °C overnight. The cells were inoculated into a YPD medium at the dilution of 1:250 and grown at 30 °C for 37 h. Cells were diluted into fresh YPD to maintain continuous growth over the course of the experiment.

Miscellaneous Procedures

CCO activity was measured by TMPD assay (McEwen et al.,

1985). Mitochondrial DNA was visualized by DAPI staining (Williamson and Fennell, 1979). Western blot analysis, SDS-PAGE (Sambrook et al., 1989) and yeast cell transformation were conducted according to standard protocols.

Antiserum

Antiserums used for this study were generously provided by the following laboratories; Cytochrome c oxidase complex (Poyton), Succinate Dehydrogenase Complex (Fanger), Cytochrome bc1 Complex (Fanger, Jenson), ATPase Complex (Neupert), Mia1p (Cumsky), Pet191p (J. McEwen), Tim23p and Tim17p (Jensen), Tim44p (Pfanner), mHsp60 (Haley), mHsp70 and Porin (Schatz).

Results and Discussion

Mia1p Depletion Impairs Cell Growth

The role of *MIA1* in non-mutant yeast cells was studied by investigating the effect of Mia1p depletion over time. *MIA1* encodes an essential gene product; thus it is expected that Mia1p-depleted cells will display impaired growth. A system was designed in which *MIA1* could be conditionally expressed by making a construct, pgal*MIA1*, in which the native promoter of *MIA1* was replaced by the promoter from the GAL1 gene. The plasmid was then introduced into a yeast strain (*Dmia1*) that lacked the *MIA1* gene. In this system, the *MIA1* gene on the plasmid can be selectively expressed when cells are grown in galactose media and alternately repressed when the cells are grown in glucose media. Growth was tested by patching wildtype, p*MIA1*, and pgal*MIA1* cells onto YPGal and YPD plates. Wild-type and *Dmia1* cells containing p*MIA1* were used as positive controls. The results showed that the cells containing the pgal*MIA* plasmid exhibited impaired growth on the glucose media (YPD) as compared to growth on galactose media (YPGal) (Figure 1). This result was as expected: the plasmid was not expressed (turned off) on glucose media and was expressed (turned on) on galactose media. The impaired growth of the *Dmia1* cells containing pgal*MIA1* suggests that Mia1p was depleted when cells were grown on glucose.

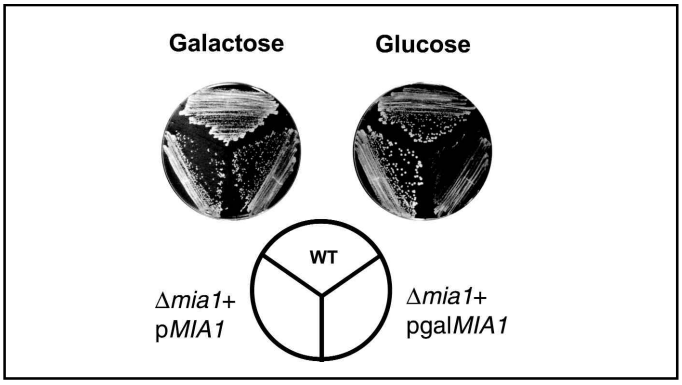

Figure 1
Mia1p depletion impairs cell growth. Wild-type (W303.1a) or *Dmia1* cells containing either p*MIA1* or pgal*MIA1* were patched onto galactose (YPGal) or glucose (YPD) media and grown for 3 days at 30 °C.

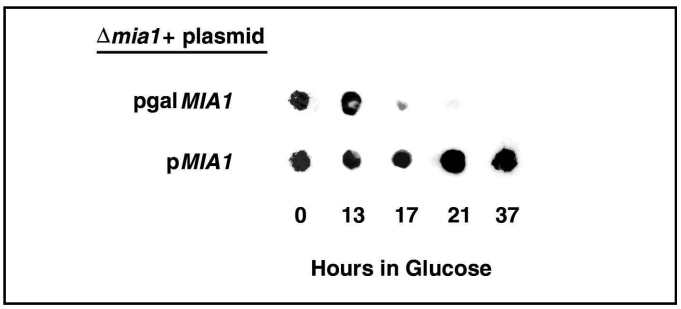

Figure 2
Mia1p depletion causes loss of CCO activity. CCO activity measured by TMPD assay. An equal number of cells were spotted on paper, lysed and treated with TMPD.

Mia1p Depletion Causes Loss of Cytochrome c Oxidase Activity

The *MIA1* gene product was previously identified as a suppressor of the mutant protein, COX5a109R, of subunit Va of CCO. This mutant causes a defect in CCO assembly and results in a severe decrease in respiratory function (manuscript in preparation). Therefore, it would be probable that a loss of Mia1p would have an effect on CCO activity. Cells containing either p*MIA1* or pgal*MIA1* were grown in conditions that would deplete Mia1p (as described in Materials and Methods). CCO activity was measured by TMPD assay at various intervals from 0 to 37 h following the switch from galactose to glucose media. The results indicate that in cells containing pgal*MIA1*, CCO activity was decreased by 17 h and undetectable by 37 h (Figure 2). Western blot analysis of mitochondria from pgal*MIA1*-containing cells at 37 h depletion showed greatly reduced levels of Mia1p in comparison to levels in mitochondria from YPD-grown cells containing wild-type Mia1p (Figure 2). Long exposure of the blots showed that a small amount of Mia1p was still present (data not shown). This low level of Mia1p expression is apparently sufficient to support slow growth of cells containing pgal*MIA1*, as seen in the plate assay, and probably resulted from incomplete repression of the GAL1 promoter under these conditions.

Mia1p Depletion Causes Loss of Respiratory Proteins/Complexes

Because it was determined that depletion of Mia1p results in a decrease in CCO activity, the next experimental step was to determine whether the effect is specific to subunit Va or

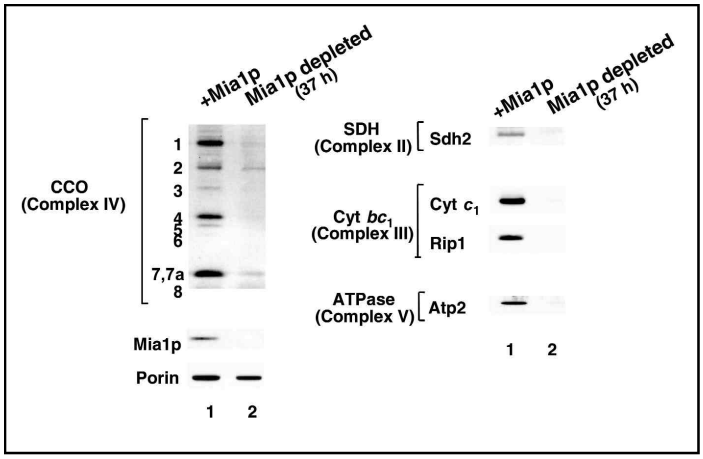

Figure 3
Mia1p depletion causes loss of respiratory proteins/complexes. Western blot analysis was used to measure the steady state levels of respiratory complexes using antiserum specific to various mitochondrial proteins as indicated.

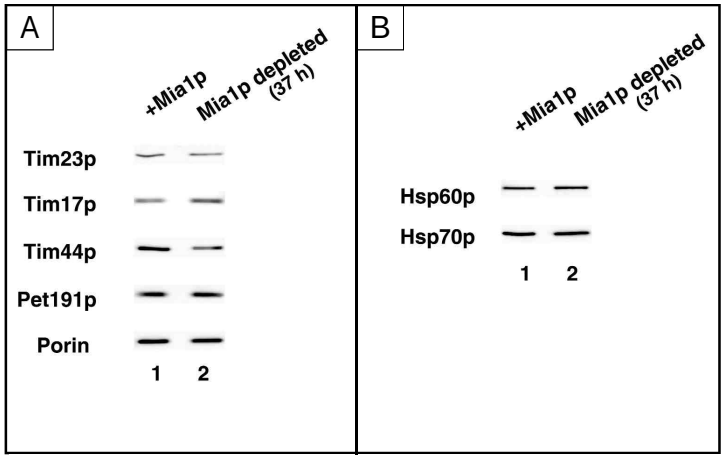

Figure 4
Mia1p depletion does not affect other mitochondrial proteins/complexes. Western blot analysis was used to measure the steady state levels of respiratory complexes using antiserum specific to various mitochondrial inner membranes (A) and matrix proteins (B). Porin was used as a loading control.

if other CCO subunits are affected. To determine the effect of Mia1p depletion on the CCO complex, Western blot analysis was conducted on mitochondrial proteins using antiserum specific to the CCO holoenzyme. Mitochondria were prepared from *Dmia1* cells containing either pgal*MIA1* or wild-type *MIA1* after 37 h of growth in YPD. Results show that in Mia1p-depleted cells, all subunits of the CCO complex are effected and protein levels are all reduced to a similar extent (Figure 3). It can be concluded that the effect of Mia1p depletion is not specific to subunit Va but has a similar affect on all subunits of the CCO complex.

The next aim of the study was to ascertain whether the effect of Mia1p depletion is specific for the CCO complex or if other respiratory complexes are similarly affected. Western blot analysis was carried out on mitochondria prepared from *Dmia1* cells containing either pgal*MIA1* or wild-type *MIA1*, again after 37 h of growth in YPD. This blot used antiserum specific to certain subunits of Succinate Dehydrogenase (Respiratory Complex II), Cytochrome bc1 (Respiratory Complex III), and ATPase (Respiratory Complex V). Results indicate that in Mia1p-depleted cells, the levels of all respiratory complex subunits are similarly reduced (Figure 4A). It can be concluded that Mia1p depletion affects not only the CCO complex, but other respiratory complexes as well.

Mia1p Depletion Does Not Affect Other Mitochondrial Proteins/ Complexes

After determining that Mia1p depletion causes loss of several respiratory complexes, experiments were performed to determine the general effect of Mia1p depletion on inner mitochondrial membrane and matrix proteins/complexes. Western blot analysis was performed on cells using antiserum specific to the inner membrane proteins Tim23p, Tim17p, Tim44p, and Pet191p, and to matrix proteins Hsp60p and Hsp70p. Analysis revealed that the levels of inner membrane and matrix proteins are not reduced (Figure 4). This indicates that Mia1p depletion does not have an effect on all inner membrane proteins. The proteins Tim23p, Tim17p, Tim44p, and Hsp70p are all part of an inner membrane pore complex through which nuclear encoded proteins, including subunit Va of CCO, are imported into the mitochondria. Since the levels of these proteins are not affected, Mia1p depletion may not affect the levels of all multiprotein complexes of the inner membrane. Experiments are in progress to test the function of the inner membrane pore complex in Mia1p-depleted mitochondria.

Mia1p-Depleted Cells Display Altered Mitochondrial Morphologies

To further investigate the function of Mia1p in the mitochondria, the cells were examined for changes in cell morphology and stained with DAPI to visualize chromosomal and mitochondrial DNA (mtDNA) (Williamson and Fennell, 1979). *S. cerevisiae* cells contain approximately 25-50 copies of mtDNA packaged into 10-30 DNA/protein complexes called nucleiods (Williamson and Fennel, 1979). It was expected that these nucleoids and the nucleus would be brightly stained. Mia1p-depleted cells were examined by DAPI staining at 0, 13, 17, 21, and 37 h after transfer to glucose media. Wild-type cells showed staining of nuclear DNA (large spot) and mitochondrial DNA nucleoids (punc-

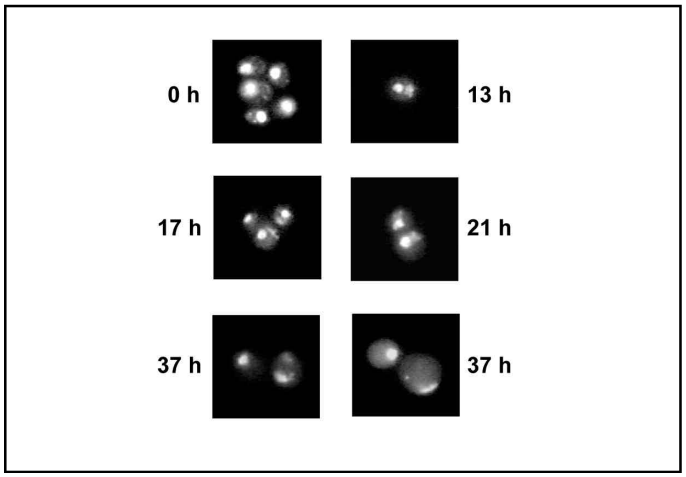

Figure 5
Mia1p-depleted cells display altered mitochondrial morphologies. Cells were fixed and stained with DAPI to visualize chromosomal and mitochondrial DNA. WT cells (0 h) show staining of nuclear DNA (large spot) and mitochondrial DNA (punctate staining in cytosol). Cells were grown on glucose and assayed at 13, 17, 21 and 37 h. By 37 h, 43% were rho– (lose mitochondrial DNA), 33% had aggregated mitochondrial DNA and 18% were enlarged.

tate staining in cytosol) (Figure 5). Mia1p-depleted cells exhibited two different populations in addition to the wild-type cells; there were cells that had lost mitochondrial DNA (rho$^-$ cells), and cells with aggregated mtDNA (nucleoids). The presence of rho$^-$ cells and cells with aggregated nucleoids increased over time so that by 37 h, 43% of the cells were rho$^-$ and 33% had nucleoids. In addition, 18% of the cells were enlarged to 2-3 times the normal size. The implication of this finding is not immediately apparent because there are many plausible explanations for the enlargement of cells. The observation at 17 h that only 23% of the depleted cells were rho$^-$, at which time CCO activity was virtually undetectable, is consistent with the hypothesis that loss of respiratory function preceded loss of mtDNA. Further studies are required to see if loss of respiratory activity is related to the loss of mtDNA in rho$^-$ cells. The formation of aggregated mtDNA (nucleoids) in Mia1p-depleted cells may eventually result in cells losing mtDNA altogether (rho$^-$).

Conclusion

The goal of this project was to investigate the role of essential gene *MIA1* in yeast mitochondria. This study showed that cells depleted of Mia1p lose CCO activity, generate rho$^-$ cells (lose mitochondrial DNA), display altered mitochondrial morphology, and lose respiratory proteins/complexes. It can be concluded that Mia1p is likely involved in import or assembly of respiratory complexes in mitochondria. Further experiments are in progress to investigate the role of Mia1p in respiratory functions.

The results of the current study and ongoing experiments will contribute to a better understanding of the import and localization of CCO subunits and other respiratory complexes in the inner mitochondrial membrane. Many proteins important in mitochondrial functions in yeast are evolutionarily conserved and have homologs in humans. These human homologs are often functionally interchangeable with the yeast protein (Beal et al., 2000). There are several known neurodegenerative diseases in humans with nuclear gene defects that affect mitochondria. These include Friedreich's ataxia, Wilson's disease, hereditary spastic paraplegia, deafness-dystonia, and Leigh's disease (Beal et al., 2000). The *MIA1* gene has a human homologue, CG1-136, with 65% similarity; therefore, it is probable that defects in the *MIA1* human homolog will also result in severe disease. The further understanding of mitochondrial biogenesis may help to develop therapies to address such problems.

Acknowledgements

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