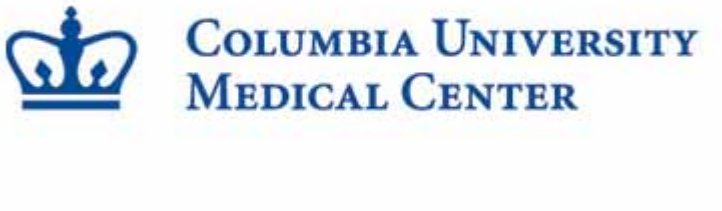

Elucidation of the role of the MICOS complex in signal transduction from mitochondrial DNA nucleoids

Master thesis

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Abstract

The MICOS complex protein MicF has recently been identified to play a vital role in the mitochondrial DNA inheritance checkpoint, a control system that halts cell cycle progression in cells lacking mitochondrial DNA (mtDNA). We show in this report that another member of the complex, MicA, also plays an important role in the mtDNA inheritance checkpoint as its deletion led to cell cycle progression in cells lacking mtDNA (Rho^0 cells). We observed that deletion of MicA, just like deletion of MicF, led to aggregated mtDNA in addition to the severe defects in the architecture of the inner mitochondrial membrane observed in all MICOS deletion strains. These findings lead us to believe that MicA and MicF might be involved in the signal transduction from mtDNA across the mitochondrial membranes and that their deletion leads to loss of contact sites between the membranes and thereby renders transduction across the membranes impossible.

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1. Introduction

Severe neurodegenerative diseases like Alzheimer's and Parkinson's disease are incurable and continue to plague millions of people worldwide each day. At the same time, metabolic diseases like diabetes type II are a continually growing global health problem. These are a few examples of diseases that have all been linked to a common source: mitochondria [**1**] [**2**].

Already in 1956, Otto Warburg discovered a link between abnormal mitochondrial behavior and cancer. He found that cancer cells even in presence of oxygen exhibited high levels of glycolysis and low mitochondrial respiratory activity [**3**]. Observations have been made that the copy number of mitochondrial DNA (mtDNA) is altered in every cancer form studied. Further studies have shown that dysregulation of the mtDNA checkpoint alters its copy number, possibly suggesting its involvement in the Warburg effect [**4**]. Moreover, mutations of the mtDNA have been shown to possibly lead to defects in heart and skeletal muscles in addition to being involved in aging and the age-related neurodegenerative diseases previously mentioned [**5**].

The mitochondrial contact site and cristae organizing system (MICOS) complex, a protein complex present in the inner mitochondrial membrane, has been shown to be vital for the maintenance of mtDNA as well as properly functioning respiratory activity due to its maintenance of the inner membrane architecture [**6**] [**7**]. Interestingly, studies carried out by Pallavi Srivastava of the Pon lab showed that deletion of one of the MICOS components led to bypass of the mtDNA inheritance checkpoint, indicating its role in the signal transduction from the mtDNA nucleoids in the mitochondrial matrix to the cytosol (Unpublished data). Up to this point, little is however known about the possible involvement of the remaining members of the MICOS complex.

The goal of this study is therefore to map out the function of the remaining components of the MICOS complex and trying to determine their possible involvement in mediating signal transduction from the mitochondrial matrix to the outer mitochondrial membrane, thereby elucidating their possible roles in the mtDNA inheritance checkpoint.

In order to do so, yeast of the species *Saccharomyces cerevisiae,* also known as baker's yeast, was used as a model organism. Particularly, its short replicative lifespan allows us to more efficiently evaluate growth rate and cell cycle progression. Due to the nature of the project, where numerous transformations are required, yeast is an excellent model organism, since it is easily genetically manipulated. Importantly, it also has many similarities to human cells and higher eukaryotes in terms of cellular organization.

2. Theoretical background

Mitochondria

The mitochondrion was likely first discovered in the 1840s, when the first intracellular constituents were seen. The ability to carry out oxidative phosphorylation makes mitochondrion the powerhouse of the eukaryotic cell. Although being what it is most commonly known for, providing energy is far from its only function. The mitochondrion has also been shown to play a crucial role in apoptosis [**8**] and is responsible for biosynthesis of vital cellular components such as fatty acids and steroid hormones [**9**] [**10**]. The mitochondrion is evidently a vital constituent of our cells and maintenance of healthy mitochondria thereby of utmost importance.

The mitochondrion exhibits a complex organization, shown to be vital for its proper functioning. It consists of an inner and an outer membrane, which collaborate in carrying out essential functions such as protein import [**11**]. As it carries out oxidative phosphorylation, it also comprises all of the sub complexes of the respiratory complex as well as its own DNA, simply called mitochondrial DNA or mtDNA. The inner membrane exhibits a very distinct architecture, which has been shown to be important for properly functioning mitochondria. It is divided into three subsections; the inner boundary membrane (IBM), the cristae junctions (CJs) and the cristae. As can be seen in figure 1 below, the cristae are invaginations that greatly increase the surface area of the inner membrane. They have been shown to be highly enriched in sub complexes of the respiratory complex, and mutations affecting the cristae have been shown to lead to grave impairment of the respiratory activity of the cell [**12**]. The inner membrane is bent to form cristae at the cristae junctions. The contact sites, abbreviated CS in the figure, are sections of the inner boundary membrane enriched in proteins responsible for vital functions such as protein import [**13**]. The mitochondrial DNA is located in close proximity to these contact sites in the mitochondrial matrix [**6**].

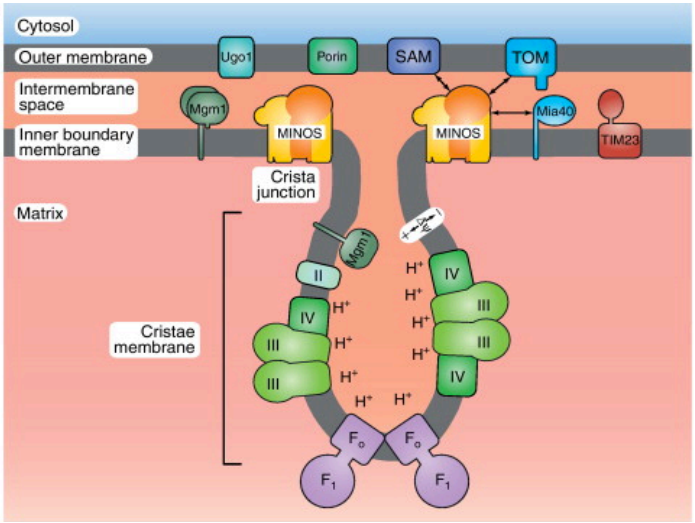


Figure 1. Cristae and the MICOS complex.
Cristae are invaginations that greatly increase the surface area of the inner membrane. The MICOS complex (previously known as MINOS complex) is located on each side of the cristae [14].

MtDNA

Mitochondrial DNA of *S. cerevisiae* is roughly 85.8 kB in size, with usually between 20-100 copies per yeast cell [**15**]. It has been seen to localize to punctate structures uniformly distributed along the mitochondria called mtDNA nucleoids**.** MtDNA contains sequences encoding vital subunits of the respiratory complex, thereby playing an important part in the formation of a functional respiratory complex (figure 2) [**16**]. The mtDNA however only encodes only 37 of the roughly 3000 genes needed for constructing a mitochondrion [**17**].

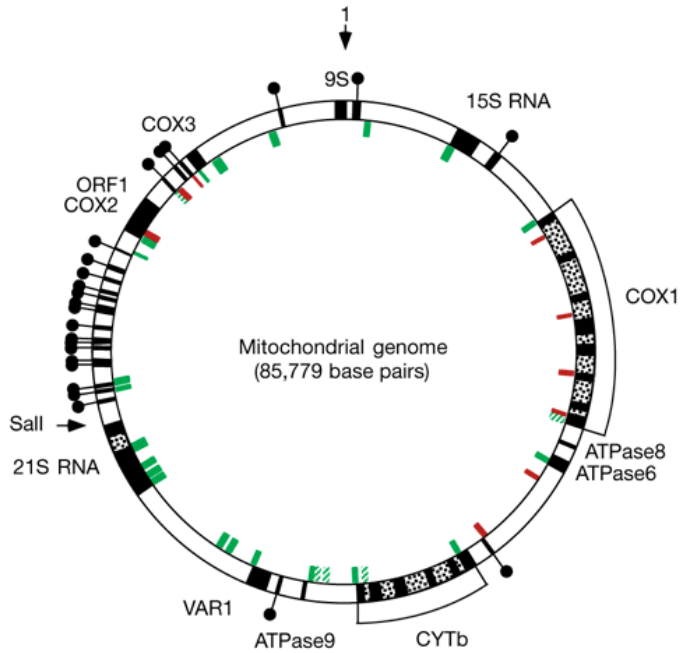


Figure 2. MtDNA of *Saccharomyces Cerevisiae* [18].

Yeast cells have the ability survive without mtDNA through anaerobic respiration, where the energy provision is shifted from oxidative phosphorylation to production of ATP through glycolysis [**19**].

Several severe diseases and conditions have been coupled to changes in mtDNA. However, due to factors such as the presence of numerous mtDNA copies in each cell, a different inheritance mechanism compared to nuclear DNA (mtDNA is maternally inherited) and a belief that mtDNA disease rarely occurs, knowledge within the field has up until recent years been relatively limited [**20**]. Later years increased research has however lead to identification of an increasing number of diseases coupled to changes in mtDNA, and mitochondrial functional decline in elderly has for example been related to age-related diabetes type II [**21**] as well as observations of increased somatic mtDNA mutations in human primary cancers [**22**]. Furthermore, the mere presence of mtDNA has been shown to play an important role in a cell cycle checkpoint called the mtDNA inheritance checkpoint [**4**].

Cell cycle checkpoints

The most thoroughly studied checkpoints have been shown to act by three main mediators: a sensor, which detects a certain unwanted alteration in a cell, a signal transduction pathway and an effector, which can halt the progress of the cell cycle, hopefully repair the damage and if not so trigger cell death [**23**]. Cell cycle checkpoints are best characterized in mammalian cells. As an example, cell cycle arrest in the G_1/S checkpoint is initiated by the inactivation of Cdc25A. The mentioned protein works by cancelling the inhibitory phosphorylation of CDK2 (cyclin-dependent kinase 2), which activity is required for cell cycle progression [**24**]. The arrest is subsequently prolonged through the induction of p53 activity [**25**]. p53 promotes transcription of several genes, such as a cyclin-dependent kinase inhibitor (CKI) called p21, which stages cyclin-dependent kinase (CDK) inhibition and thereby cell cycle arrest [**26**].

Previous studies in the Pon laboratory revealed a checkpoint that responds to loss of mtDNA. They showed that deletion of mtDNA resulted in inhibition of cell cycle progression from G1 to S phase in the cell division cycle. To verify that the result was not due to loss of genes encoded by the mtDNA, experiments were conducted where native mtDNA was replaced with non-coding DNA, which showed cell cycle progression [**4**]. This implicates that the mere presence of mtDNA plays an important role in the checkpoint. Furthermore, it was observed that Rad53p, the yeast homologue to the mammalian protein kinase Chk2 involved in the DNA damage checkpoint, plays a regulatory role in the mtDNA inheritance checkpoint [**4**]. The mechanism of signal transduction from the mtDNA nucleoids to the nucleus however remained unclear, and cells lacking mtDNA (called Rho^0 cells) were thereby vital for gaining further understanding of the checkpoint.

Knowledge about the sensor of the mtDNA inheritance checkpoint was scarce, but studies conducted by Pallavi Srivastava of the Pon lab showed that deletion of a member of the MICOS complex called MicF led Rho^0 cells to bypass the checkpoint (Unpublished data). In other words, due to the deletion of this MICOS component they progressed in the cell cycle in spite of their lack of mtDNA. This discovery led to further interest about the functions of the rest of the MICOS complex and their possible involvement in the checkpoint, and that is why the MICOS complex has been in focus in this project.

The MICOS complex

The MICOS (mitochondrial contact site and cristae organizing system) complex is responsible for the organization of cristae and the inner mitochondrial membrane [**27**]. As was shown previously, the inner mitochondrial membrane comprises a highly elaborate architecture, for which the MICOS complex plays an important role [**28**] [**29**]. It has been shown to be of importance for formation of cristae junctions, which in turn is critical for mitochondrial respiratory activity, for formation of contact sites between mitochondrial outer and inner membranes and for maintenance of mtDNA [**30**] [**31**]. The mtDNA is located in close proximity to the contact sites in the mitochondrial matrix.

The MICOS complex consists of six, or possibly seven members as a novel protein and a possible new member has recently have discovered. These are MicA, MicB, MicC, MicD, MicE, MicF and the novel protein, which so far goes under the name of F2. Recent reports have also suggested that Cox17 in yeast and Qil1 in humans also interact with the MICOS complex [**32**] [**33**].

It has recently been shown that the members of the MICOS complex are further divided into two sub complexes; one comprising MicA, MicB and MicE and the other MicF and MicC [**7**]. Out of the two sub complexes it has furthermore been suggested that MicA and MicF functions as key players in the assembly and functions of their respective sub complex [**27**] [**29**] [**34**].

The members of the complex are further described below, where they are divided according to the mentioned sub complexes.

MicF

The most studied protein and a crucial component of the MICOS complex is MicF. As mentioned, studies carried out by Pallavi Srivastava of the Pon lab showed that MicF-deletions in Rho^0 cells led to bypass of the mtDNA inheritance checkpoint, indicating that the protein might be involved in the signal transduction from mtDNA necessary to initiate cell cycle arrest (Unpublished data).

This suggestion is strengthened by observations that MicF interacts both with the SAM and the TOM complex of the outer membrane as well as with mtDNA [**27**] [**34**] [**35**], opening up the possibility that MicF is responsible for the transfer of signal from mtDNA nucleoids to the outer mitochondrial membrane in the checkpoint.

The Nunnari lab also showed in a MICOS deletion strain, in which every component of the MICOS complex was knocked out except for MicF, that it has the ability to independently localize to substructures of the inner mitochondrial membrane and thereby probably to self-assemble [**7**]. Looking closer into these localizations, it was seen that it was in close proximity to the respiratory complexes and cristae markers Qcr2 and Atp2, suggesting that MicF furthermore marks cristae junctions [**7**].

MicC

MicF is accompanied by MicC in one of the sub complexes, and the former has been shown to be required for MicC stability. Even though MicC is considered part of the MicF sub complex, it was discovered that MicC has a functional relationship to both of the MICOS sub complexes; MicC has been shown to disperse MicF foci in the MICOS knockout strain, as only 20% of the cells displayed MicF-EGFP foci with MicC present as compared to 80% when only MicC was absent. The absence of MicC also specifically resulted in the formation of MicE focal assemblies in cells. It has thereby been suggested that MicC, the only member of the MICOS complex which is localized to the inter membrane space between the two mitochondrial membranes, operates as an assembly regulator of the sub complexes. This hypothesis was evaluated by mass spectrometry of MicF purifications from both the mentioned MICOS deletion strain and wild type. MicF lost almost all of its interactions with the rest of the MICOS complex in the absence of MicC, further strengthening the suggestion that it works as an assembly regulator of the sub complexes [**7**].

Studies conducted in mammalian cells showed that the same scenario could be true there, as knockdown of MicC resulted in a highly reduced amount of an intact MICOS complex. Its knockdown was also seen to lead to highly disrupted formation of cristae [**36**].

MicA

MicA, together with MicF has been found to be conserved within a large amount of eukaryotes, while the conservation of most other MICOS components is restricted to the opisthokonts [**37**]. Deletion of MicA has furthermore been shown to lead to severe alterations of mitochondrial morphology and is believed to be a key player within the MICOS complex, together with MicF [**29**].

MicA was shown to be responsible for altering the inner mitochondrial membrane by bending the cristae into their characteristic hairpin loop structures at the cristae junctions by homo-oligomerizing. Two glycine-rich motifs of the protein enable this homo-oligomerization [**38**]. These findings were confirmed by another research team, which had similar observations [**39**]. They also found that overexpression of MicA led to highly elongated and disrupted cristae. The only other MICOS component that showed cristae alterations as a consequence of overexpression was MicF, but this overexpression showed a different cristae ultrastructure [**39**].

MicE

MicE has been shown to be dependent on MicA for stability, as its steady-state levels were reduced by ~80% *micA*Δ cells [**29**].

Similar experiments to those carried out to determine the localization of MicF in the MICOS deletion strain have been carried out to determine the localization of the MicA/MicE/MicB sub complex in a *micFΔ* strain. The results showed that this sub complex seems to have the ability to localize to mark cristae junctions, independent of MicF [**7**].

MicB

MicB has been shown to function in supporting the formation and stability of its sub complex [**7**]. It is however worth to note that MicB is the only established member of the MICOS complex that does not have a human homologue [**29**].

MicD

MicD is a relatively obscure protein which has not been focused on a lot, possibly because it is paralogous to MicE. Recent studies however shed some light on the human form of the protein by showing that MicD interacts with MicA, MicE and MicF. They also saw that MicD is antagonistically regulated by MICE and coregulated with MicA. This possibly suggests that these two paralogue proteins have partially overlapping or redundant functions [**40**].

F2

F2 is a novel protein discovered in the Pon laboratory, which was indicated by genome-wide screens that it has the capacity to interact with MicF. Pallavi Srivastava from the Pon lab found that the gene encoding F2 is up-regulated in yeast that exhibit tight mtDNA checkpoint control. She also found that it localizes to mitochondria and

that rho^0 cells bearing deletion in F2 bypass the mtDNA inheritance checkpoint (Unpublished data). This raised the interesting possibility that F2 is a possible component of the MICOS complex that contributes to the mtDNA inheritance checkpoint control.

3. Materials and Methods

Yeast Growth Conditions

S. Cerevisiae of the genetic background BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) from Open Biosystems (Huntsville, AL) was used for construction of all recombinant strains used in the experiments (Supplemental table 1). Synthetic Complete (SC) medium was used unless otherwise indicated. Cells were grown in 5 mL of medium in 50 mL Falcon conical bottom tubes in a shaking incubator at 30°C. Cells were grown to mid-log phase (OD_{600} = 0.1 - 0.3) prior to all experiments.

Strain Construction and deletion of mtDNA

S. cerevisiae strains used in this study are summarized in Supplemental Table 1. All yeast strains were created in the BY4741 wild-type background by homologous recombination according to a standard lithium acetate protocol (R. D. Gietz, R. H. Schiestl, A. R. Willems, and R. A. Woods, "Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure," Yeast, vol. 11, pp. 355-60, Apr 15 1995) [**41**]. Deletion strains were created by disrupting genomic locus of MICOS genes in WT and cells expressing Tom70p-GFP and Cit1p-mcherry by transformation with a PCR-amplified cassette from pFA6a-KanMX6 containing the kanMX selectable marker or PCR-amplified sequence containing LEU2 derived from the pOM cassette system. *MICOS* genes were tagged at its C-terminus with GFP by transformation with a PCR-amplified GFP tagging cassette from the pFA6a-GFP system (Addgene, Cambridge, MA) containing *HIS3* and 40–base pair homology to the endogenous locus. Disruption of a gene was verified using PCR amplification

Rho^0 derivatives were generated from wild type by two consecutive 1-d treatments of 25 µM EtBr (Goldring et al., 1971) [**42**]. Rho^0 cells were confirmed by lack of growth on plates containing a non fermentable carbon source and absence of mtDNA with DAPI staining.

Microscopy

Images were collected with a microscope (Axioskop 2; Carl Zeiss) equipped with a 100×/1.4 Plan-Apochromat objective (Zeiss, Thornwood, NY) and a cooled charge-coupled device camera (ORCA-1; Hamamatsu Photonics) at 25°C. For visualization of GFP, mCherry, and DAPI, fluorophores were excited by a mercury arc lamp and imaged through a motorized filter wheel using the following filters: GFP (excitation 482/28, emission 525/50), mCherry (excitation 545/25, emission 632/60), and DAPI (excitation 402/15, emission 455/50). Hardware was controlled by Nikon Elements software. Z stacks were obtained in 13 steps at 0.5 µm intervals, and images were finally deconvolved using a constrained iterative restoration algorithm in Volocity 5.5 (PerkinElmer).

Growth rate analysis

All strains of both Rho^+ and Rho^0 were grown to mid-log phase and transferred to a sterile 96-well plate. A growth curve analysis assay was run for all of the strains using a Tecan Infinite® 200 NanoQuant (Tecan US, Morrisville, NC) measuring the optical density at 600nm every 20 minutes for 72 hours. The plate reader was operated using Magellan software.

Flow cytometric cell cycle analysis

Cell cycle analysis was conducted in brief through the following procedure: Cells were synchronized by adding α-factor at G1 stage. Cells then were collected at 0, 20, 40, 60, 80, 100, 120, 150 and 180 minutes, fixed with ethanol and subsequently stained with propidium iodide and analyzed in a fluorescence-activated cell analyzer (LSR II; BD). The amount of cells in every stage of the cell cycle was finally determined using the FlowJo software (Tree Star). Fold change was determined by comparing the amount of cells having progressed from G1 from time point zero.

4. Hypothesis

MtDNA resides within the mitochondrial matrix and is tightly associated with mitochondrial membranes at sites of close contact between the mitochondrial outer and inner membranes. However, the checkpoint machinery that inhibits cell cycle progression in response to loss of mtDNA resides within the nucleus.

One fundamental question is how information regarding mtDNA content can be transmitted from the mitochondrial matrix to the cytosol and ultimately to the nucleus. We propose that this signal is transmitted across contact sites between outer and inner membranes. Indeed, our finding that deletion of MICF, which results in loss of contact sites, bypasses the mtDNA inheritance checkpoint. Based on these findings, we hypothesized that one or more MICOS members are involved in the signal transduction from mtDNA nucleoids in the mtDNA inheritance checkpoint in addition to MicF.

Based on the findings that the novel protein F2 interacts with MicF and that *f2Δ* Rho^0 cells also bypass the mtDNA inheritance checkpoint, we also hypothesized that F2 additionally interacts with other MICOS components.

5. Results and discussion

Deletion of MICOS proteins affects mitochondrial morphology

To assess the effects of deletion of the MICOS components on mitochondrial morphology, they were deleted in a yeast strain expressing Tom70-GFP and Cit1-mcherry. Tom70p is an outer membrane protein and Cit1p is localized to the mitochondrial matrix, which allowed us to efficiently examine the effects through fluorescence microscopy.

We found that, in accordance to what has been reported earlier, all of the deletion strains show high amounts of lamellar mitochondrial morphology compared to the reticular mitochondria seen in wild type. According to expectations, we saw that the suggested key players MicA and MicF knockouts exhibited very high levels of lamellar mitochondria (80% in *micAΔ* and 85% in *micFΔ*) [**29**].

micCΔ and *micEΔ* cells however surprisingly showed even higher degrees of lamellar mitochondria (93% in both *micCΔ* and *micEΔ*) than previously reported (75% in both *micCΔ* and *micEΔ*) [**29**].

The relatively low amounts of lamellar mitochondria seen in *micBΔ*and *micDΔ* (63% and 64% respectively) were however not a surprise, given that they are suggested to play peripheral roles in the complex. The exhibited amounts are similar to observations made in previous reports, but slightly higher amounts of lamellar mitochondria were found in *micBΔ* cells (30%) [**29**].

Our deletion strains exhibited higher amounts of lamellar mitochondria than reported earlier. This is because of two reasons: First, we used only fluorescence microscopy to examine the defect in mitochondrial morphology while Hoppins et al., 2011 [**29**] have used Electron microscopy to study discrepancies in mitochondrial architecture. Second, we have quantified a relatively small number of cells (n=20) for these experiments. Imaging more cells (n>100) will provide more accurate account of effect of MICOS deletion. Nonetheless, our results suggest that all of the members of the MICOS complex play important roles in maintaining the architecture of mitochondria.

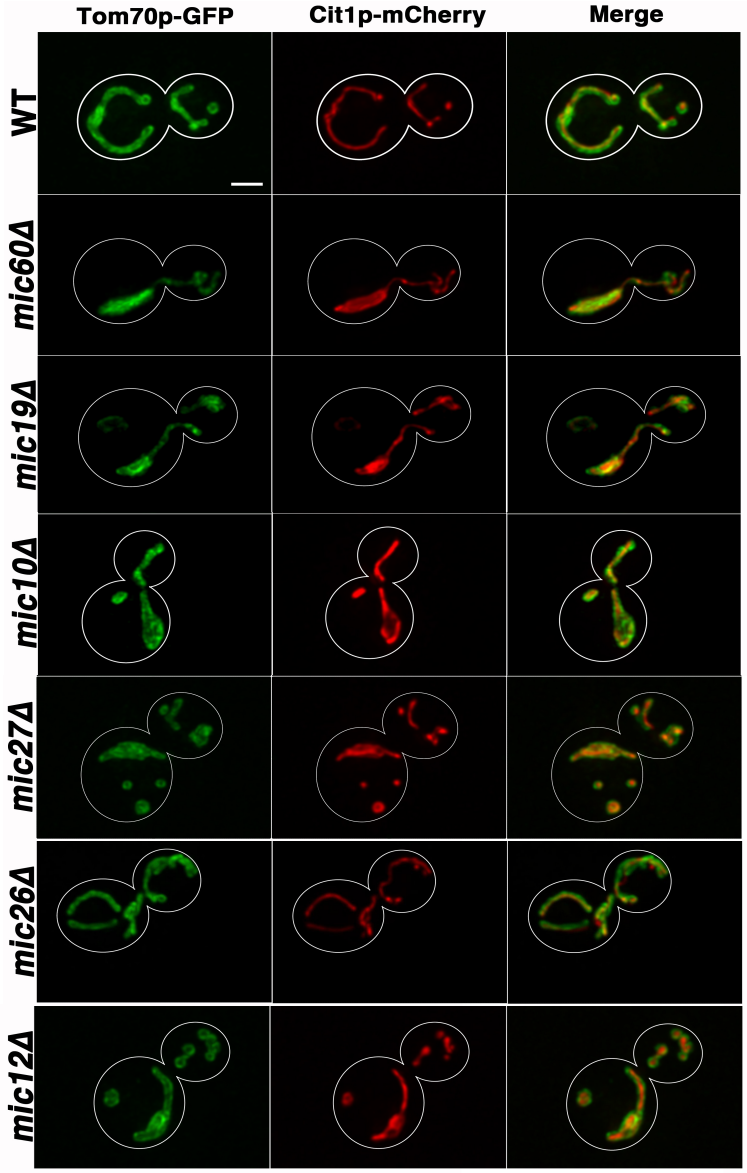


Figure 3. Deletion of MICOS proteins affects mitochondrial morphology.
Representative images of matrix targeted mCherry and outer membrane targeted GFP wild type and indicated deletion strains.

MICOS mutants exhibit altered mtDNA morphology and distribution

As mentioned in the introduction, mtDNA is normally distributed in punctate structures along the inner mitochondrial membrane. The MICOS complex has been shown to be vital for the maintenance of mtDNA, as certain MICOS mutants have been shown to exhibit aggregated nucleoids of decreased number and increased size [**6**]. Again, most studies have focused on MicF, which has been shown to be vital for mtDNA maintenance. Its role in mtDNA maintenance is due to what was observed in a recent study, that MicF plays a part in the architecture of the mtDNA D-loop [**35**]. MicA has also been shown to be important for mtDNA maintenance, while *micBΔ, micCΔ, micDΔ* and *micEΔ* showed no effect on mtDNA [**6**].

MICOS deletion strains were stained with DAPI for 15 minutes, which was enough time to stain the mitochondrial DNA but not to penetrate the nucleus and stain the nuclear DNA. We found that MicA and MicF seem to be crucial for mtDNA maintenance, since highly aggregated mtDNA nucleoids were observed in the cells in micA and micF knockouts (90% in *micAΔ* and 100% in *micFΔ*). This is in accordance with previous studies [**6**].

In *micEΔ* we saw ambiguous results, as 50% of the mtDNA nucleoids were overly aggregated in this knockout. The remaining MICOS protein knockouts showed little or no effects on the mtDNA nucleoids compared to wild type (10% in *micBΔ* and *micDΔ* and 0% in *micCΔ*). This was no surprise in the case of *micCΔ,* since it is the only MICOS protein which is solely located to the inter membrane space, whereas all the other members of the complex exhibit at least segments in both the inter membrane space and the inner membrane [**39**]. The relatively high amount of overly aggregated mtDNA seen in *micEΔ* was unexpected and in contrast to previous studies, where none of the MICOS protein knockouts except MicA and MicF were shown to have any effect on mtDNA maintenance [**6**]. This is possibly due to the time sensitivity of DAPI staining in this case leading to unexpected results.

In summary, these results further strengthens earlier research and the suggestion that MicA and MicF are the key players of the MICOS complex, as they are not only very important for maintenance of the inner membrane architecture but also for maintenance of mtDNA.

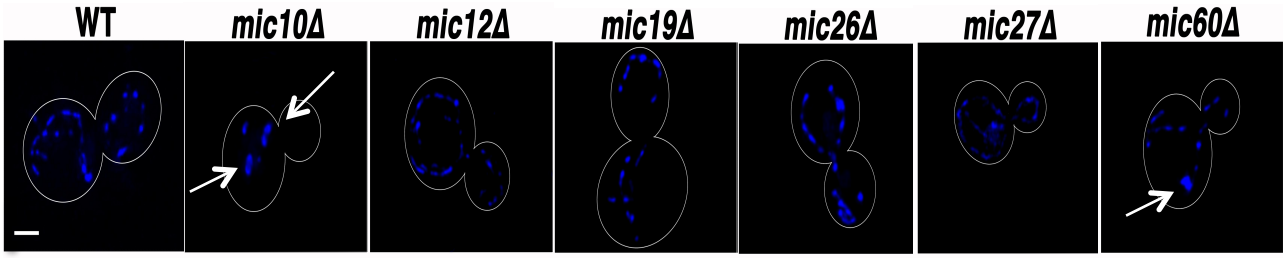


Figure 4. MICOS mutants exhibit altered mtDNA morphology and distribution.
DAPI staining revealed that *micAΔ* and *micFΔ* exhibit high amount of overly aggregated mtDNA. Representative images are shown for wild type and the indicated deletion strains. Arrows point to examples of aggregated mtDNA nucleoids.

micFΔ and *micAΔ* Rho^{0}s bypass the mtDNA inheritance checkpoint

Flow cytometric cell cycle analysis had not been carried out in MICOS mutants previous to these trials, except for the *micFΔ* trial carried out by Pallavi Srivastava, and they were the core of this project since the results give good indications whether they play important roles in the mtDNA inheritance checkpoint.

The analysis was conducted by synchronizing the cells at G1 stage, followed by releasing them into the cell cycle and subsequently collecting the cells at different time points and fixing them with ethanol. The cells were then stained with DNA binding dye, propidium iodide, and DNA content was quantified by flow cytometery. We have also examined the growth rate of wild type and all MICOS deletion strains with (rho$^+$) or without mtDNA (rho^0). The growth curve analysis was carried out by growing cells in rich growth medium (YPD) and measuring the optical density every 20 minuted for 72 hours.

Growth and cell cycle progression of wild type was analysed and used as a reference that MICOS mutants could be compared to. As expected, wild type Rho$^+$ exhibited a steady growth rate and cell cycle progression and the results were thereby used as a reference in each MICOS deletion analysis.

We found that *micAΔ* Rho^0 progressed in the cell cycle and thereby bypassed the mtDNA inheritance checkpoint. The growth curve analysis showed that *micAΔ* Rho^0 cells exhibit a growth rate similar to the rho$^+$ cells, which further strengthened the finding that *micAΔ* Rho^0 cells do not arrest at G1/S stage in the cell cycle (Figure 5). These results are in line with the other experiments, in which MicA has also proven to be a key player, and it suggests that MicA plays an important role also in the checkpoint.

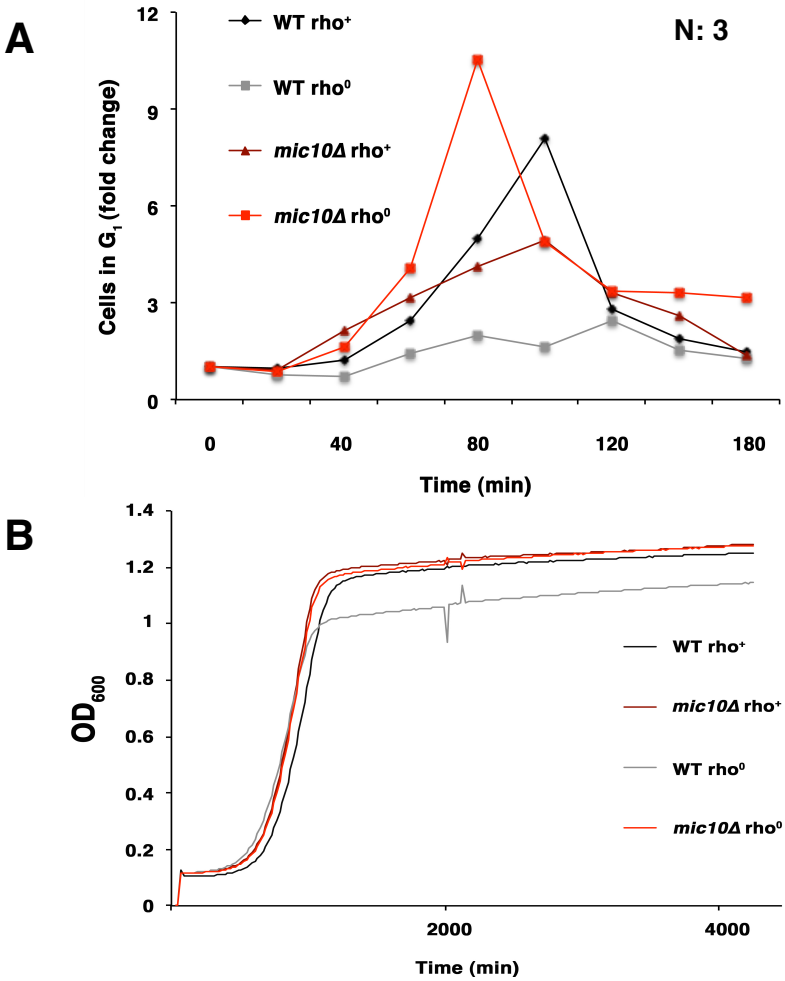


Figure 5. *micAΔ* bypasses the mtDNA inheritance checkpoint.
(A) Cell cycle analysis showing progress for *micAΔ* in the cell cycle.
(B) Growth curve for *micAΔ*, showing steady growth close to that of wild type Rho$^+$.

micBΔ Rho^0 does not seem to bypass the mtDNA inheritance checkpoint. *micBΔ* Rho$^+$ and *micBΔ* Rho^0 progress in the cell cycle similarly to the wild type Rho$^+$ and wild type Rho^0, respectively. The growth rate of the *micBΔ* cells is similar to that of wild type (Figure 6).

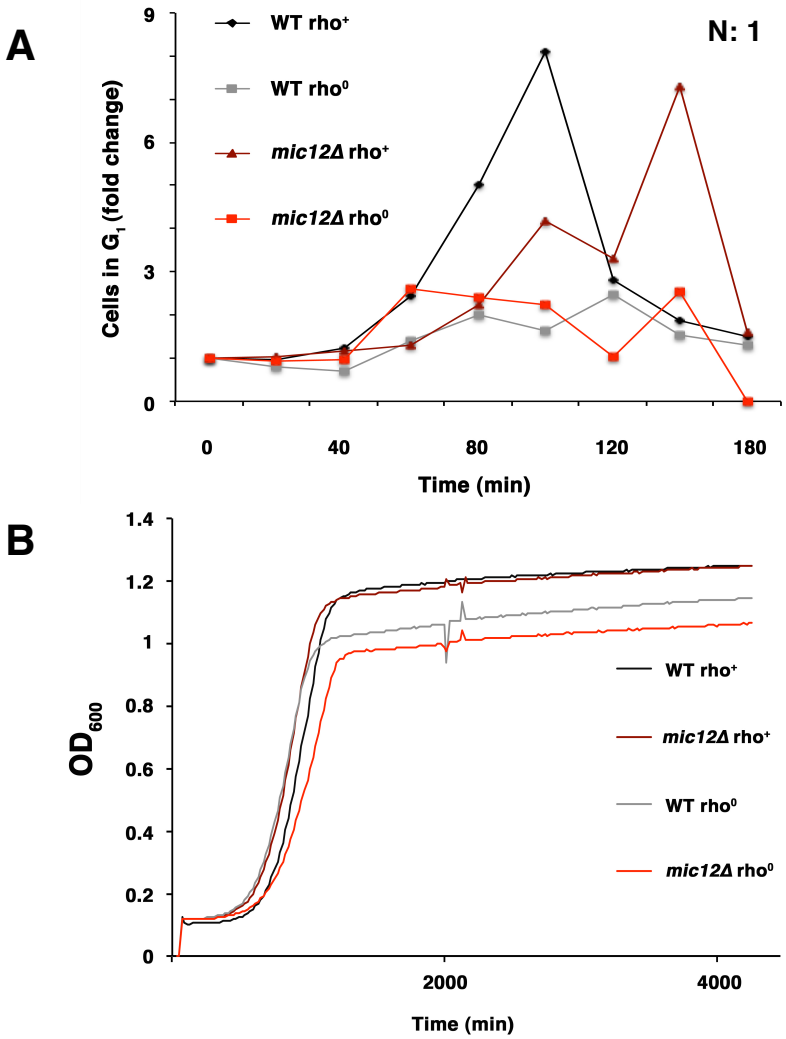


Figure 6. *micBΔ* does not bypass the mtDNA inheritance checkpoint.
(A) Cell cycle analysis showing cell cycle arrest for *micBΔ* Rho^0 in the cell cycle.
(B) Growth curve for *micBΔ,* showing limited growth close to that of wild type Rho^0.

micCΔ Rho^0 neither bypassed the mtDNA inheritane checkpoint. The growth curve strengthened this result, as this strain was seen to be highly growth deficient (Figure 7).

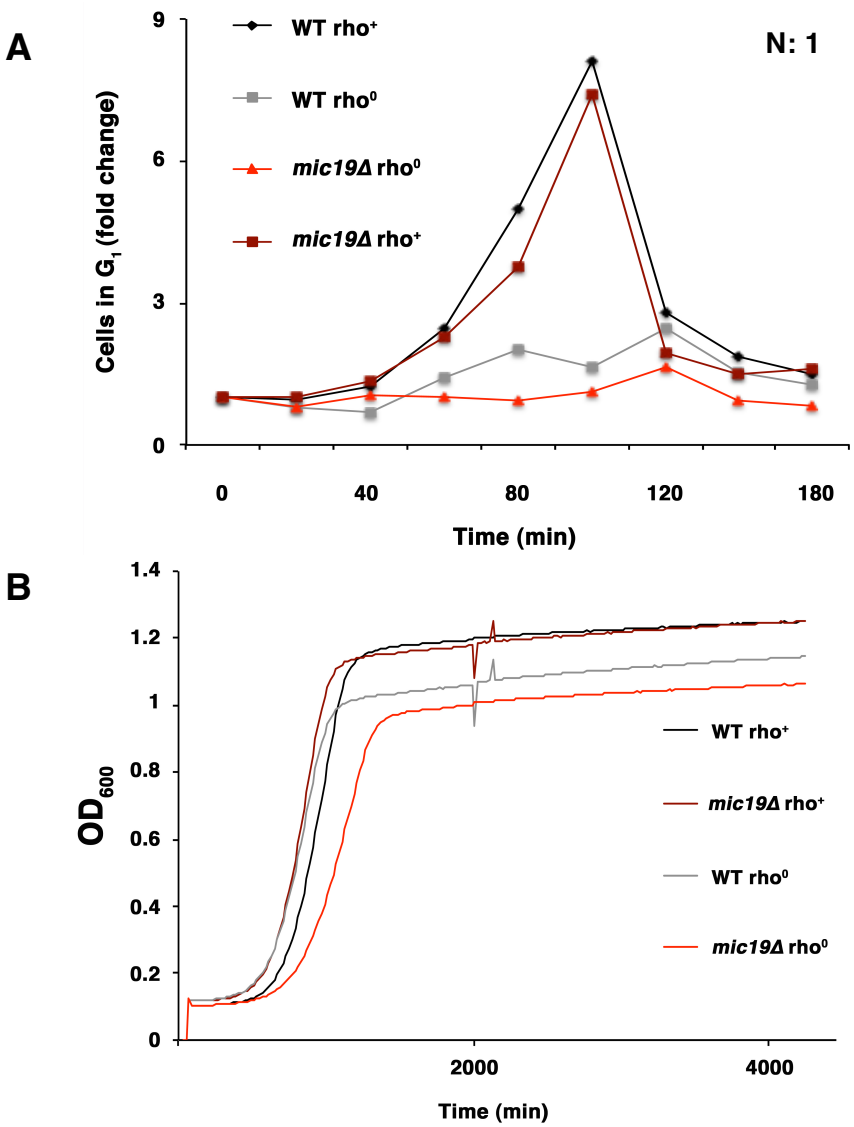


Figure 7. *micCΔ* does not bypass the mtDNA inheritance checkpoint.
(A) Cell cycle analysis showing cell cycle arrest for *micCΔ* Rho^0 in the cell cycle.
(B) Growth curve for *micCΔ,* showing limited growth close to that of wild type Rho^0.

In *micDΔ* strains, both Rho$^+$ and Rho^0 were found to be arrested at G1/S stage. This is a very surprising result as *micDΔ* Rho$^+$ cells do not show significant defects/loss of mtDNA organization. Growth rate of *micDΔ* Rho^0 is however as expected significantly lower than that of *micDΔ* Rho$^+$. Further investigation is required to completely understand the role of micD in cell cycle progression.

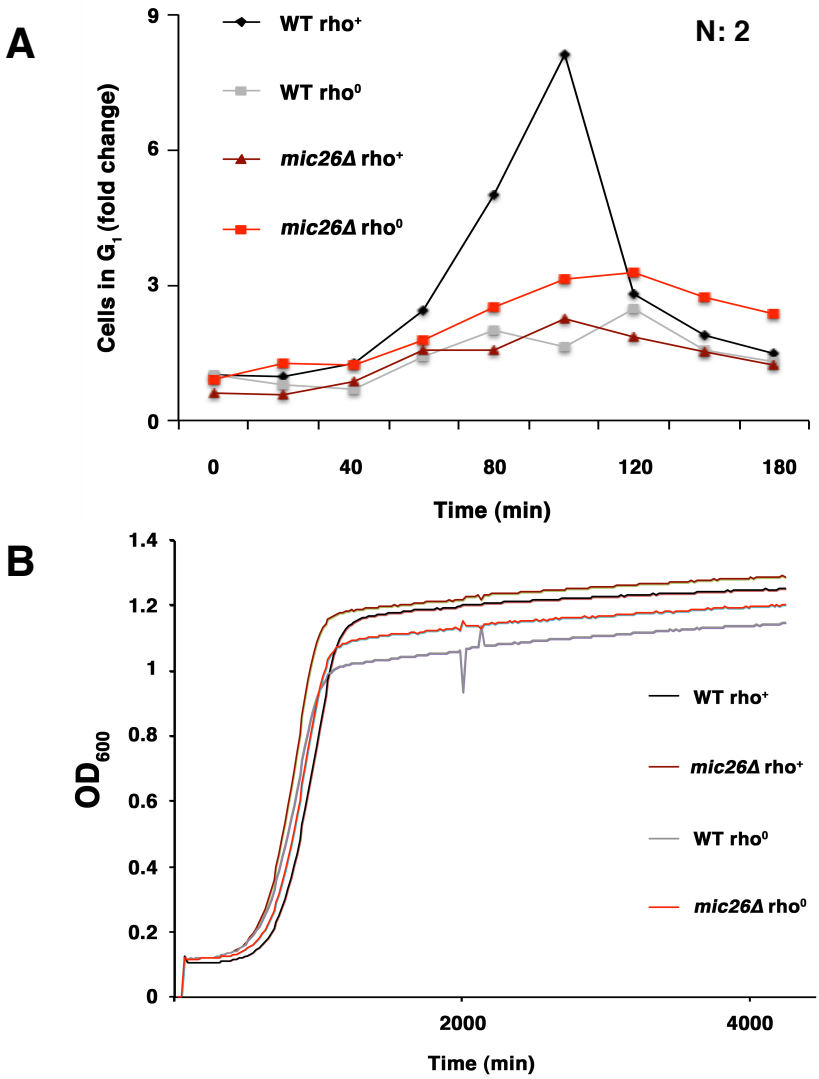


Figure 8. *micDΔ* does not bypass the mtDNA inheritance checkpoint.
(A) Cell cycle analysis showing cell cycle arrest for both *micDΔ* Rho^0 and *micDΔ* Rho$^+$ in the cell cycle.
(B) Growth curve for *micDΔ,* showing slightly limited growth of *mic26Δ* Rho^0.

micEΔ Rho$^+$ and *micEΔ* Rho^0 cells have progressed in the cell cycle but at a lower rate that of wild type Rho$^+$ cells. *micEΔ* Rho^0 cells however displayed defect in growth rate in comparison to *micEΔ* Rho$^+$ cells. This partial bypass observed in the *micEΔ* cells cannot be explained from our current understanding of this matter and requires further investigation (Figure 9).

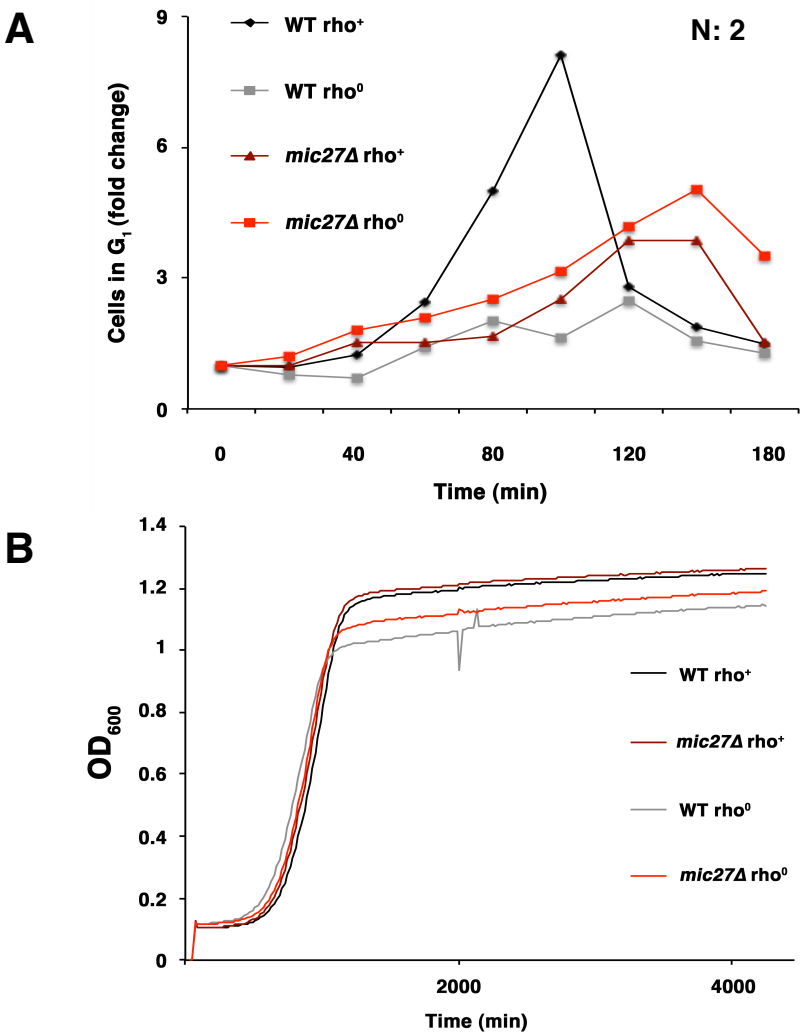


Figure 9. *micEΔ* does bypass the mtDNA inheritance checkpoint.
(A) Cell cycle analysis indicating possible bypass of the mtDNA inheritance checkpoint for both *micEΔ* Rho^0 and *micEΔ* Rho$^+$.
(B) Growth curve for *micEΔ*, showing slightly limited growth of *micEΔ* Rho^0.

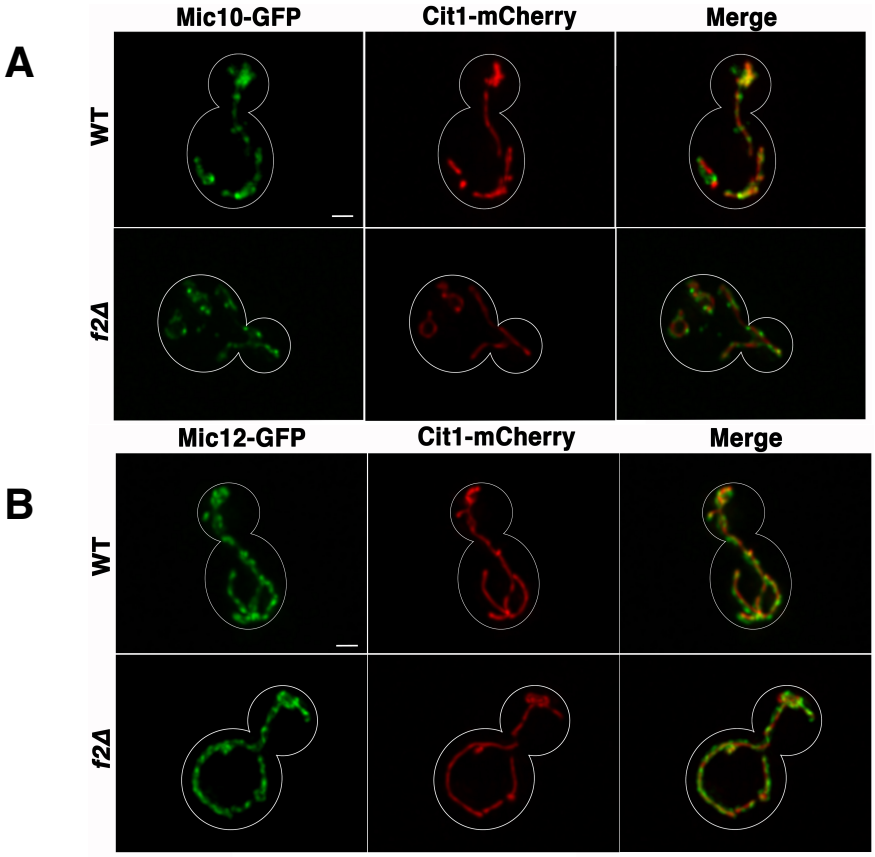
Altogether, our results indicate that MicA in addition to MicF plays a vital role in the mtDNA inheritance checkpoint, which has never been shown before. Since the MicE Rho^0 knockout also showed a potential bypass of the checkpoint, this protein should be further studied to establish its role in the checkpoint. In addition, the Rho$^+$ knockouts of MicD and MicE showed somewhat confusing results, as they did not progress in the cell cycle. None of the other MICOS proteins are indicated to be crucial for maintaining a functional checkpoint.

F2 is not required for MICOS localization

Previous studies by Pallavi Srivastava indicate that MicF is required for normal localization of F2, as deletion of MICF results in decrease in the amount of F2 that localizes to mitochondria. We speculate that F2 may be a novel protein of the MICOS complex, so we decided to study its possible interactions with other MICOS proteins.

To test this, we decided to microscopically examine GFP-tagged MICOS proteins in a F2 deletion strain and determine whether the MICOS proteins localize to mitochondria properly compared to wild type.

MICOS proteins localize to punctate structures that co-localize with mitochondria. We find that deletion of F2 does not have any obvious effect on the localization or distribution of all MICOS proteins studied (Figure 11).



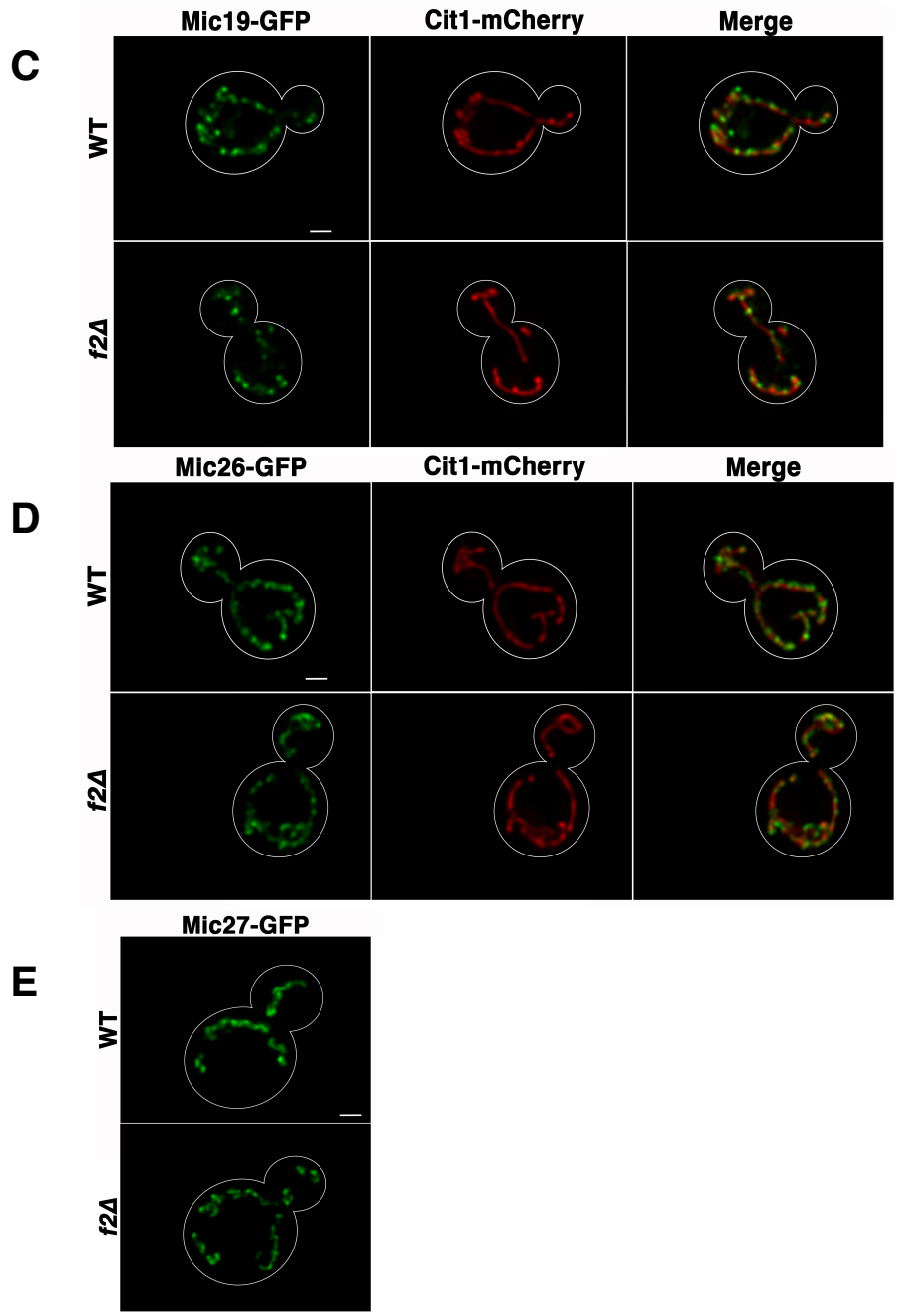


Figure 10. F2 is not required for MICOS localization.
(A) MicA was distributed in a similarly focal-like pattern also in the absence of F2.
(B) No effects on the distribution of MicB could be seen in the absence of F2.
(C) Neither the distribution of MicC was affected by the F2 deletion
(D) No effects on the distribution of MicD could be seen in the F2 deletion strain.
(E) MicE distribution was unaffected by the deletion of F2.

F2 does not require MicD or MicE for localization

Corresponding experiments were made for the opposite situation, i.e. examination of GFP-tagged F2 in *micDΔ* and *micEΔ* cells. F2 was found to localize to bright foci in a pattern along the mitochondria in wild type, similar to what was observed for the MICOS proteins. Examination of F2-GFP in the remaining MICOS knockout strains did unfortunately not fit the time frame of the project and should be carried out in order to obtain the whole picture.

In contrast to the earlier observations that F2 requires MicF for localization, foci of F2-GFP was not seen to vary significantly between *micDΔ, micEΔ* and wild type (Figure 12).

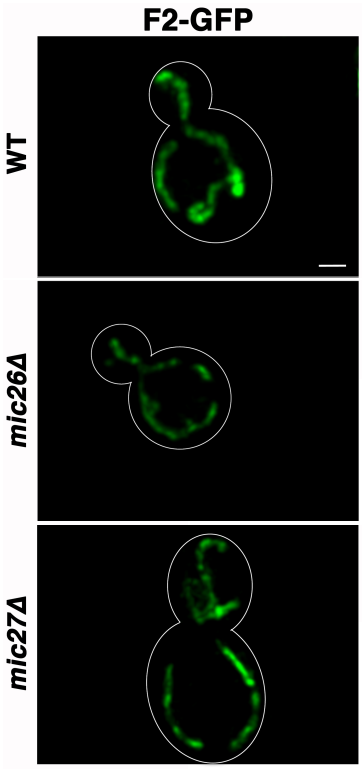


Figure 12. F2 does not require MicD or MicE for localization.
No effects on F2 distribution could be seen in any of the studied knockouts.

Altogether we found that none of the established MICOS proteins localized differently compared to wild type in the *f2Δ* cells. Neither did we observe that F2 localized differently in the absence of MicD and MicE.

4. Conclusions and Future directions

We have seen that MicA and MicF seem to be the most important constituents of the MICOS complex, and that they both play vital roles in the signal transduction from mtDNA nucleoids to the outside of mitochondria. We speculate that MicF might be either directly or indirectly responsible for mediation of the signal from the mtDNA nucleoids, as it has been seen that MicF physically interacts with both mtDNA [**35**] and the SAM and the TOM complex of the outer mitochondrial membrane [**27**] [**34**]. We also speculate that MicA, which plays a vital role by bending the inner membrane to form cristae by homo-oligomerizing, is responsible for maintaining the contact sites without which signal transduction would be impaired.

Table 1. Summary of the results from studies on mitochondrial morphology, mtDNA aggregation and mtDNA inheritance checkpoint arrest of the MICOS deletions.

Strain	Defects in mitochondrial morphology (% of cells analyzed)	Aggregated mtDNA nucleoids (% of cells analyzed)	MtDNA inheritance checkpoint arrest
WT	0	0	+
micAΔ	80	90	-
micBΔ	63	10	+
micCΔ	93	0	+
micDΔ	64	10	?
micEΔ	93	50	?
micFΔ	85	100	-

Altogether, we find that all of the members of the MICOS complex affect the mitochondrial morphology but only MicA, MicF and possibly MicE affect mtDNA nucleoid structure. Our data indicate that the role of MICOS proteins in controlling the mitochondrial architecture might not be important for its ability for signal transduction from the mtDNA. We however observed a correlation between the MICOS proteins involved in mtDNA nucleoid morphology and bypass of the mtDNA inheritance checkpoint.

We gained some insights on how signal transduction from the mtDNA nucleoids to the outside of the mitochondria is carried out in the mtDNA inheritance checkpoint, but how the signal from the surface of the mitochondria through the cytosol to the nucleus is carried out remains to be elucidated.

We also obtained preliminary evidence through live cell microscopy that F2 interacts with and requires MicA for localization, in addition to MicF. Further research should focus on determining the role of MicA in F2 localization. Possible interactions with other MICOS complex proteins should also be researched in order to determine its role within the complex.

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Appendix

Supplemental table 2. Strains used in the project.

Strain name	Parent(s)	Genotype
MHY001	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 micBΔ::KANMX
MHY002	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 micBΔ::KANMX
MHY003	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 micBΔ::KANMX tom70 GFP::his3Δ1 cit1mcherry::hygro
MHY004	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 micBΔ::KANMX tom70 GFP::his3Δ1 cit1mcherry::hygro
MHY005	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 micEΔ::KANMX tom70 GFP::his3Δ1 cit1mcherry::hygro
MHY006	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 micCΔ::KANMX
MHY007	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 micCΔ::KANMX
MHY008	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 micCΔ::KANMX tom70 GFP::his3Δ1 cit1mcherry::hygro
MHY009	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 micEΔ::KANMX
MHY010	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 micBΔ::KANMX Rho0
MHY011	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 WT::KANMX Rho0
MHY012	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 micCΔ::KANMX Rho0
MHY013	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 micAΔ::POM13
MHY014	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 micAΔ::POM13 tom70 GFP::his3Δ1 cit1mcherry::hygro
MHY015	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 micDΔ::KANMX
MHY016	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 micDΔ::KANMX tom70 GFP::his3Δ1 cit1mcherry::hygro
MHY017	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 micFΔ::POM13
MHY018	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 micCΔ::POM13 Rho0
MHY019	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 micDΔ::KANMX Rho0
MHY020	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 micEΔ::KANMX Rho0
MHY021	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 micFΔ::POM13 Rho0
MHY022	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 micCΔ::KANMX Rho0
MHY023	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 micEΔ::KANMX
MHY024	BY4741	
MHY025	BY4741	
MHY026	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 MicAGFP::HIS
MHY027	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 MicAGFP::HIS
MHY028	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 fjrp2Δ::KANMX MicAGFP::HIS
MHY029	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 MicBGFP::HIS
MHY030	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 fjrp2Δ::KANMX MicBGFP::HIS
MHY031	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 fjrp2Δ::KANMX MicBGFP::HIS
MHY032	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 MicCGFP::HIS
MHY033	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 fjrp2Δ::KANMX MicCGFP::HIS
MHY034	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 fjrp2Δ::KANMX MicCGFP::HIS
MHY035	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 MicDGFP::HIS
MHY036	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 MicDGFP::HIS

MHY037	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 fjrp2Δ::KANMX MicDGFP::HIS
MHY038	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 MicEGFP::HIS
MHY039	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 MicEGFP::HIS
MHY040	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 fjrp2Δ::KANMX MicEGFP::HIS
MHY041	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 fjrp2Δ::KANMX MicEGFP::HIS
MHY042	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 micAΔ::POM13 fjrp2GFP::HIS
MHY043	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 micAΔ::POM13 fjrp2GFP::HIS
MHY044	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 micBΔ::POM13 fjrp2GFP::HIS
MHY045	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 micBΔ::POM13 fjrp2GFP::HIS
MHY046	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 micDΔ::POM13 fjrp2GFP::HIS
MHY047	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 micEΔ::POM13 fjrp2GFP::HIS
MHY048	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 micEΔ::POM13 fjrp2GFP::HIS
MHY049	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 micBΔ::POM13 fjrp2GFP::HIS
MHY050	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 fjrp2Δ::KANMX MicEGFP::HIS
MHY051	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 MicEGFP::HIS
MHY052	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 micEΔ::POM13 fjrp2GFP::HIS

Supplemental table 3. Primers used during the project

Purpose	Plasmid	Sequence
Deletion of MICA	pFA6a	CTACGAGAGGGAATAAACACGGAAAAAGACAAAAT ATACCCGGATCCCCGGGTTAATTAA
Deletion of MICA	pFA6a	TTTTTTTTTTTGAATATATATAAAGCATCGTCGCTTAA GAGAATTCGAGCTCGTTTAAAC
Deletion of MICB	pFA6a	CAGACAGTGGACTAAGAACCAGCAGATAACGGAGAG AATCCGATCCCCGGGTTAATTAA
Deletion of MICB	pFA6a	CATGAGGATGTTCGTTACAGTAGGAGAAATAGAAAGC TCGGAATTCGAGCTCGTTTAAAC
Deletion of MICC	pFA6a	ACAAAAACAAGGTGGTATATCGACTAATACAGAGTCAATC CGGATCCCCGGGTTAATTAA
Deletion of MICC	pFA6a	CGAATTCTTTTTGGTCGAGTTTATGTATACTTTTTCTTAT GAATTCGAGCTCGTTTAAAC
Deletion of MICD	pFA6a	ATACAAAAATAACTACTGTATTTGATATAGCACGGAAACA CGGATCCCCGGGTTAATTAA
Deletion of MICD	pFA6a	GTATTTATAAGATGAATAAGCGCCAGGTGCTAAACAGAGT GAATTCGAGCTCGTTTAAAC
Deletion of MICE	pFA6a	AGACAGAAGCAGCACACCATTTACCAATACAGCTTCCAAA CGGATCCCCGGGTTAATTAA
Deletion of MICE	pFA6a	ATGGACATGATAATGAACAAAAAAAGATATCCGCTTGATA GAATTCGAGCTCGTTTAAAC
PCR control for MICC		TCAATCATCAATTAAATAAT
PCR control for MICC		TCATGACACCAAGAGAACAA
PCR control for MICD		GATGATGGATGATGATGAAG
PCR control for MICD		AAGACTCAAGCATTCAAAAA
PCR control for MICE		ATTGCAAGCATGCTTTATAC

PCR control for MICE		AATTAATGTA CTACAAACG A
PCR control for MICB		GGACTTATCGATGATTTCGT
PCR control for MICB		TTTCAGCTCAGCCAATTCAA
PCR control for MICA		TGAATATAAGGAGCGGGTAT
PCR control for MICA		AATTTTCGTTTTCAGATGTG
Deletion of MICA	Pom	CTTTGCTACGAGAGGGAATAAACACGGAAAAAGACAAAATAT ACC TGCAGGTCGACAACCCTTAAT
Deletion of MICA	Pom	TATTATTTTTTTTTTTGAATATATATAAAGCATCGTCGCTTA AGA GCAGCGTACGGATATCACCTA
Deletion of MICD	Pom	AGGATATACAAAAATAACTACTGTATTTGATATAGCACGGAA ACA TGCAGGTCGACAACCCTTAAT
Deletion of MICD	Pom	TGTATGTATTTATAAGATGAATAAGCGCCAGGTGCTAAACAG AGT GCAGCGTACGGATATCACCTA