Investigating Factors that Influence Subcellular Mitochondrial Dynamics

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Abstract

Mitochondria are the powerhouse of the cell, providing energy (ATP) to support dynamic cellular processes in a variety of cell types (3). ATP is rapidly consumed upon release from mitochondria, therefore must mitochondria move throughout the cytoplasm to provide localized energy at subcellular sites (3). This movement is facilitated by Miro1, an essential adaptor protein that links mitochondria to microtubule motors (**Fig 1A**) (2). The factors that regulate Miro1 to coordinate mitochondrial movement and how Miro1 responds to signals is unknown. Previous research identified that reactive oxygen species (ROS) decrease mitochondrial motility (1), therefore we chose to investigate how ROS influence Miro1 and mitochondrial dynamics. Using a gene-edited cell line expressing GFP-tagged Miro1, immunofluorescence assays, and laser scanning confocal microscopy, I quantified the effects of ROS generated in different cellular compartments on Miro1 and mitochondrial dynamics. Menadione (cytosolic ROS) was found to decrease Miro1 association with mitochondria and significantly decrease mitochondrial networking. Rotenone (mitochondrial ROS) increased Miro1 association with the mitochondria and mitochondrial networking. This project has yielded new knowledge regarding mitochondrial responses to stress and how intracellular metabolites like ROS influence Miro1 to coordinate mitochondrial dynamics. Since disrupted mitochondrial dynamics is central to numerous disease states, including neurodegeneration and tumor metastasis, these results may provide valuable insight into the role that Miro1 plays in the establishment of ROS-associated diseases.

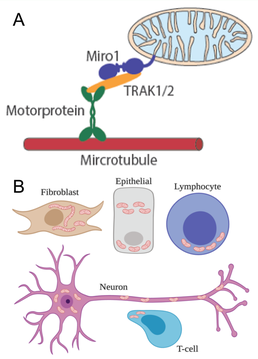

Figure 1: (A) Miro1 and other adaptor proteins link mitochondria to microtubule motors, for movement throughout the cytoplasm. **(B)** Various cell types with their unique mitochondrial positioning.

Purpose

- Identify signals that regulate Miro1
- Investigate the interaction between ROS, Miro1, and mitochondria
- Gain insight into new therapeutic avenues for cancer metastasis and other diseases

Research Questions

- How does ROS affect Miro1 association with the mitochondria?
- How does ROS regulate the size, degree of networking, directional movement, and cellular distribution of mitochondria?

Hypothesis

ROS from different sources will alter Miro1 function and lead to changes in mitochondrial structure, positioning, and networking.

Introduction and Background Information

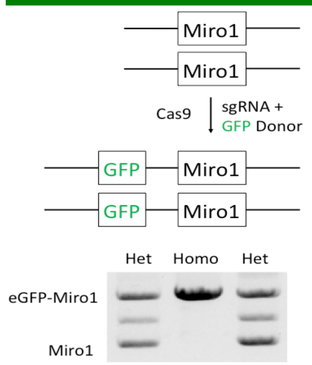

Figure 2: Generating a GFP-Miro1 cell line with CRISPR/Cas9 technology. Cas9 enzyme, small guide RNA's (sgRNA), and a GFP donor construct were introduced to MEFs to induce GFP gene insertion upstream of the Miro1 gene. Single cells were screened by polymerase chain reaction (PCR) to identify a homozygous (Homo) cell line where both Miro1 alleles have been edited to express GFP.

Miro1 controls mitochondrial movement in cells. Tools to study Miro1 are lacking, so prior to this project I assisted the Cunniff laboratory in the generation of a GFP-Miro1 cell line (**Fig 2**), allowing us to visualize Miro1 to study its response to cell signals. I used confocal microscopy to confirm the GFP fusion protein did not alter Miro1 function (**Fig 3**).

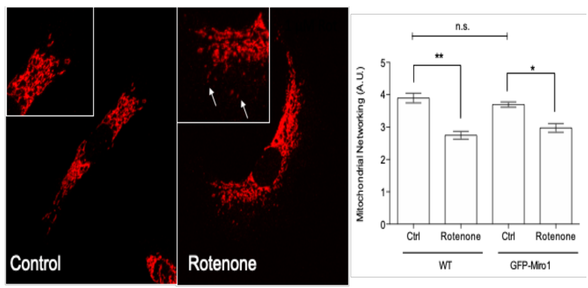

Figure 3: Comparison of control and GFP-Miro1 cells treated with 10 μM rotenone. Arrows indicate fragmented mitochondria resulting from rotenone treatment. GFP-Miro1 mitochondria had similar networking as compared to wild type cells and responded to rotenone in the same manner, indicating that Miro1 function was unaffected by the GFP fusion (* $p < 0.05$, ** $p < 0.01$, n.s.= not significant, One-way ANOVA statistical test).

Methods

1. Cell culture of GFP-Miro1 Mouse Embryonic Fibroblasts (MEFs).
2. Treatment time course with ROS generating reagents, including menadione, which increases cytoplasmic and mitochondrial ROS (1) and rotenone, which inhibits the electron transport chain increasing mitochondrial matrix ROS.
3. Cell fixation with paraformaldehyde.
4. Immunofluorescence (IF) labeling with antibodies targeting GFP-Miro1 and TOM-20 (mitochondrial protein). DAPI labeled the nucleus and Phalloidan labeled the actin cytoskeleton.
5. Imaging with the Nikon A1R Laser Scanning Confocal Microscope (4) in the UVM Microscopy Imaging Center (**Fig 4**).
6. Mitochondrial networking quantification using ImageJ software.

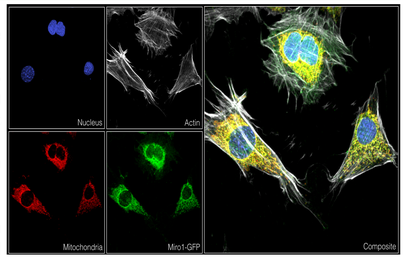

Figure 4: Images of IF labeled GFP-Miro1 MEFs were captured using a Nikon A1R laser scanning confocal microscope. Nucleus = blue, mitochondria = Red, GFP-Miro1 = Green, actin cytoskeleton = gray. As shown on the right, these images are merged to visualize association of GFP-Miro1 with mitochondria.

Results

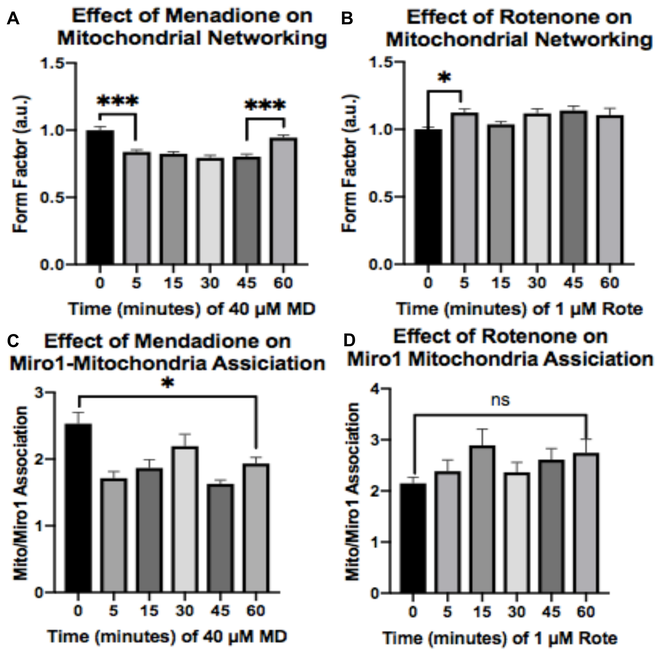

Figure 5: (**A**) Form factor, an unbiased method to measure the area and perimeter of each mitochondrion, was used to quantify mitochondrial networking. Mitochondrial networking decreased significantly in cells incubated with menadione but showed some recovery towards the end of the time course. **(B)** Mitochondrial networking increased in cells treated with rotenone. **(C)** A high-resolution pixel-by-pixel quantification was performed by our collaborator Dr. Giuseppe Di Caprio to determine the ratio of GPF-Miro1 associated with mitochondria under conditions tested. In cells incubated with menadione, the portion of Miro1 associated with mitochondria decreased over time. **(D)**. Rotenone caused a moderate increase in Miro1 association with the mitochondria. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s.= not significant, One-way ANOVA statistical test).

Discussion and Conclusion

- Using immunofluorescence and confocal microscopy, I demonstrated the effects of different ROS generating compounds on networking of mitochondria and Miro1 association.
- Menadione decreased mitochondrial networking and decreased GFP-Miro1 association with mitochondria.
- Rotenone increased mitochondrial networking and GFP-Miro1 association with mitochondria. Higher concentrations of rotenone induce mitochondrial fragmentation (**Figure 3**).
- These preliminary findings suggest ROS generated from different cellular compartments, menadione = cytosol and rotenone = mitochondrial matrix, have alternative effects on mitochondria and GFP-Miro1 dynamics.
- Changes in cellular metabolism that alter ROS levels may influence mitochondrial and Miro1 dynamics supporting ROS and mitochondrial associated diseases.

Future Experiments

- Reagents that induce other forms of cellular/metabolic stress will be investigated:

Reagent	Mode of Action
BPTES	Glutaminase 9GLS I inhibitor
Cytochalasin D	Actin polymerization inhibitor
DPI	NADPH oxidase inhibitor
Etomoxir (ETO)	Inhibitor of fatty acid oxidation
FCCP	Depolarize mitochondrial membrane potential
Glucose Oxidase	Cytoplasmic ROS inducer
Nocodazole	Microtubule binding agent (polymerase inhibitor)
Oligomycin	Mitochondrial ATP synthase inhibitor
Taxol	Suppressor of microtubule dynamics
UK5099	Inhibitor of mitochondrial pyruvate carrier

- Conduct live-cell imaging to visualize alterations to mitochondrial dynamics in real time.
- Cell fractionation will be used to isolate and purify mitochondria from cells. With protein western blotting, I will investigate the resulting distribution of Miro1 and other proteins between cellular compartments.
- Using protein immunoprecipitation of GFP-Miro1 with an anti-GFP antibody conjugated magnetic beads, I will investigate how ROS and other stressors influence protein-binding interactions of GFP-Miro1. These samples will be separated by SDS-PAGE stained with Coomassie blue and gel bands will be submitted to the UVM Proteomics Facility for protein identification by mass spectrometry.

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