Investigating the Order of Recruitment of Mitochondrial Dynamic Proteins

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Abstract

Mitochondria are an essential organelle that produces most of the cell's ATP and regulates apoptosis. Their morphology is integral to their function, and they undergo important dynamic events to maintain a healthy structure. Previous studies have shown that nodes on the endoplasmic reticulum (ER) regulate these dynamic fission and fusion events. Further, it has been shown that proteins associated with both events colocalize on the same sites. The actual mechanism of this colocalization is still largely unknown. The goal of this study was to characterize this colocalization and investigate the nature of their interactions. Through depleting cells of a fission protein (Drp1), we observed that a fusion protein (Mfn1) localization was also altered. This information provides groundwork on the nature of these proteins and how they interact. Future research into this interaction could provide insight into neurodegenerative diseases caused by inhibited mitochondrial dynamics.

Introduction

Mitochondria

Mitochondria are a membrane-bound organelle conserved across animals, fungi, and plants. They are pivotal to cell processes such as oxidative phosphorylation and intrinsic apoptosis. Their unique double membrane structure is integral to their function in both examples. The space between the two membranes, the intermembrane space, is the site for oxidative phosphorylation, where most of the ATP is produced for the cell. The inner membrane contains the proteins that make up the Electron Transport Chain (ETC), a series of protein complexes that reduce the energy potential of electrons to generate enough energy to pump hydrogen ions from the mitochondrial matrix to the intermembrane space. This process is necessary to establish a proton gradient that fuels the intermembrane complex, ATP synthase, to catalyze the formation of ATP (Ahmad et al., 2024).

Mitochondrial protein Cytochrome C normally functions within the ETC, but it is a critical signal to send the cell into apoptosis under extreme stress conditions. While Cytochrome C is not the only mitochondrial protein directly affecting apoptosis, it is the most famous example. Mitochondrial fission protein Opa1, which usually controls the size of cisternae junctions, has been shown to play a role in releasing Cytochrome C in apoptotic events (Wang & Youle, 2009).

Since most of a cell's ATP comes from the delicate structure of the mitochondrial membrane, there is a lot of incentive to keep their morphology healthy. They are incredibly dynamic organelles routinely undergoing restructuration to optimize their phenotypes. These processes are fission and fusion events; through fission, one mitochondrion is split into two, and two mitochondria are combined through fusion (Suen et al., 2008). Fusion events protect against

unhealthy mitochondria and allow elongation to optimize ATP synthesis in nutrient-deficient environments. Fission then can break down elongated mitochondria when they no longer benefit the cell. As breaking down but preserving the membrane is rather complex, many proteins are necessary for these fission and fusion events. Drp1 is a cytosolic protein that translocates to the mitochondrial membrane during mitosis and when they undergo fission events (Kashatus et al., 2011; Zhu et al., 2004). Mfn1 is a mitochondrial outer membrane protein that increases its localization to puncta at fission sites. The cycle of fission and fusion is represented in Figure 1.

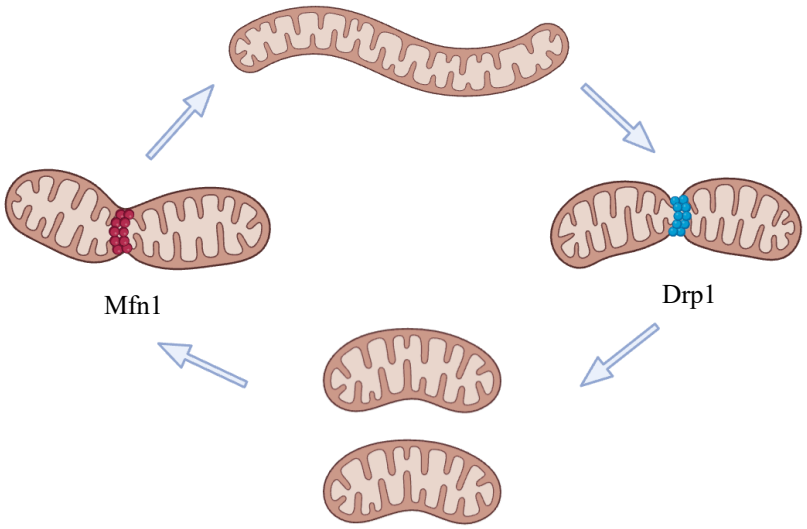


Figure 1: Depiction of mitochondrial fission and fusion cycles. Drp1 represents fission and Mfn1 represents fusion**.**

Drp1 is a GTPase which is regarded as the primary indicator of fission events (Smirnova et al., 1998). When inactive, it localizes in the cytoplasm and the general outer mitochondrial membrane but responds to membrane receptors by oligomerizing and constricting them (Wu et al., 2018). This constriction site ultimately becomes the location of the fission event when the

GTP hydrolysis event initiates the division with other integral fission proteins, such as mitochondrial fission factor (Mff) and human fission factor-1 (Fis-1) (Hall et al., 2014). Mfn1 and Mfn2 are similarly GTPases that localize to the mitochondria outer membrane. Mfn1, which will be the focus of this study, forms punctate structures at sites where two mitochondria come together at sites of fusion (H. Chen et al., 2003). Opa1 is similarly vital for fusion events but regulates the inner membrane fusion (Ishihara et al., 2006).

With mitochondria being integral to various cellular processes, multiple diseases are associated with impairments of mitochondrial dynamics. Patients lacking Drp1 have been shown to have hyper-linked mitochondrial networks and display symptoms such as microcephaly, abnormal brain development, and refractory epilepsy (Waterham et al., 2007). Additionally, mitochondrial dysfunction has been linked to neurodegenerative diseases such as Alzheimer's and Parkinson's (W. Chen et al., 2023). Both diseases have been tied to damaged dynamics and oxidative stress when they don't produce enough ATP. These cases of disease caused by mitochondrial issues highlight the relevance of this study investigating more factors in the dynamic pathways.

Endoplasmic Reticulum

The endoplasmic reticulum (ER) is a dynamic organelle made up of three distinct subcategories: nuclear envelope, sheets, and tubules (Schwarz & Blower, 2016). All three form a continuous network but perform different functions for the cell. The nuclear envelope is the most central; it forms a barrier that protects the nucleus from the rest of the cell (D'Angelo & Hetzer, 2006). It serves as a mediator, allowing mRNA out of the nucleus and transporting specific nucleus-located proteins inside while keeping everything else out (Li & Noegel, 2015). Continuous with the nuclear envelope, the ER sheets and tubules form an intertwined network of

ER throughout the cell. It is usually densest with the most sheet content centrally and thins out with mostly tubule content towards the cell periphery (Lee & Chen, 1988). ER sheets, also referred to as the rough ER, are often studded with ribosomes, carry out transmembrane protein synthesis, and are the beginning of the secretory pathway (Shibata et al., 2006). ER tubules, frequently referred to as smooth ER, are responsible for processes such as lipid and steroid synthesis and are the ER's sites of contact with other organelles (Shibata et al., 2006).

The ER tubules form contacts with other organelles in cells to coordinate and regulate various cellular activities. Namely, through this process, the ER interacts with endosomes, mitochondria, Golgi, and the plasma membrane (Wu et al., 2018). With endosomes, it has been shown that the ER is present at sites of endosome fission, where when endosomes undergo a complex maturation process, they split to traffic contents to various cellular locations. It has been shown that as endosomes move through this pathway they remain in contact with the ER (Rowland et al., 2014). Other than regulating dynamics, another important function of these contact sites is for the ER to transfer lipids produced to other organelles. The first event this was shown was that the ER transfers phosphatidylinositol-4-phosphate (PI4P), diacylglycerol, and sphingomyelin lipids to the Golgi membrane (Peretti et al., 2008). Similarly, this is also an important characteristic of plasma membrane-ER contact sites. The plasma membrane is also made up of a complex combination of lipids and sterols, which are made and transferred by the ER in order to maintain the integrity of the plasma membrane (Porter & Palade, 1957).

ER-Mitochondria Contact Sites

Most prevalent in this study, the ER forms contact sites with mitochondria to regulate their dynamic events (Friedman et al., 2011). Additionally, these contact sites are responsible for regulating lipid metabolism and calcium homeostasis (Wilson & Metzakopian, 2021). Specific

proteins are responsible for tethering the mitochondria to the ER, but the proteins involved are still largely unknown. In yeast, the ERMES complex, a collection of mitochondrial proteins (Mdm10 and Mdm34), ER protein Mmm1, and cytosolic protein Mdm12, was discovered to be a tethering complex between the two organelles (Kornmann et al., 2009). This complex is not conserved across organisms, and a similar protein complex has not yet been found in humans.

It has been shown that the ER does not just touch the mitochondria but wraps around it (Friedman et al., 2011). After wrapping, proteins involved in the constriction and dynamic events are recruited. The internal membrane is constricted first, followed by the outer membrane (Cho et al., 2017). The actual constriction event also requires actin, which is recruited before dynamic events. Drp1 is then recruited to these ER-associated mitochondrial constrictions and contributes to further constriction and eventual fission in a GTPase manner (Smirnova et al., 1998).

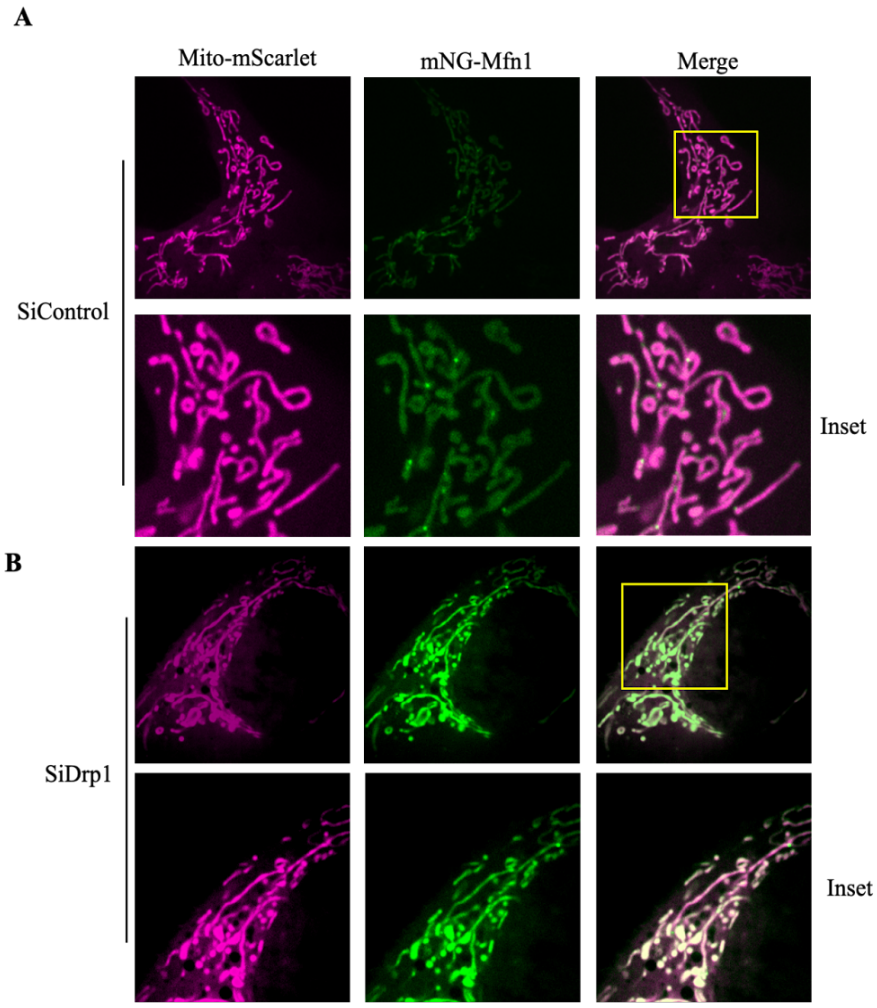
Project Justification

While it has been shown that fission and fusion machinery colocalize to the same ER sites, it has been shown that the fission machinery (Drp1) is more likely to be at sites of fusion than fusion machinery (Mfn1) at sites of fission (Abrisch et al., 2020). More generally, it is unknown why both machineries localize to the same contact sites even when only one dynamic event occurs. In this study, we aimed to identify the recruitment order of mitochondrial proteins, if the reason they occur on the same ER sites is because of the proteins having a regulatory effect on each other, and if Drp1 is more likely to be present at sites of fusion is because it is involved with recruiting fusion proteins. This will give more insight into how these dynamic events are regulated, which can further provide insight into why defects in mitochondrial dynamics cause neurological diseases.

Results

Previous studies have shown that while both Drp1 and Mfn1 localize to the same nodes, Drp1 is more likely to be present at the site of fusion events (Abrisch et al., 2020). This result indicates a hypothesis that Drp1 may be required for the recruitment of Mfn1 for fusion. To test this, I performed a siRNA knockdown of Drp1 and imaged the localization of overexpressed Mfn1 tagged with mNeonGreen. U2OS cells were plated and treated with a siRNA targeting Drp1, or a general scrambled siRNA, twice with a day in between to recover. The cells were then transfected with 75ng mNG-Mfn1, and 100ng mito-mScarlet as a general mitochondria marker. The cells were then imaged every 5 seconds, for a total of two minutes to be able to observe the dynamics of the mitochondria.

Figure 2A shows the mNG-Mfn1 localizes weakly to general mitochondria, with strong puncta at spots of mitochondrial dynamic events. The mitochondria in the control group were dynamic, and their phenotype indicates good cell health. Figure 2B shows that the mNG-Mfn1 localizes strongly to the mitochondria but without forming puncta in the Drp1 knockdown cells. The mitochondria in this population were also generally elongated compared to the control and had fewer dynamic events, which suggests that they were unhealthy. Twenty cells were analyzed to find the average number of puncta in each condition; the control cells had an average of 21.9 puncta per cell, whereas the knockdown had 5.4 puncta per cell (Figure 3C). The p-value between these two sets was 0.0034, indicating that the decrease of puncta in knockdown cells is a significant change.



C

	Control	Drp1 Knockdown
Average Punctate per cell	21.9	5.4

n = 20

Figure 2: Mfn1 localization disperses under Drp1 knockdown conditions. A: mNeon-tagged Mfn1 and mito-mScarlet were transfected and imaged in live U2OS cells that underwent a knockdown treatment with scramble siRNA. B: mNeon-tagged Mfn1 and mito-mScarlet were transfected and imaged in live U2OS cells that underwent a knockdown treatment with siRNA targeting Drp1. C: 20 cells were analyzed for how many puncta were present, and total for all cells in each condition were averaged.

To illustrate the Mfn1 localization difference between the two conditions, I took line scans of two characteristic mitochondria in both conditions. These scans measure the intensity of both channels along the mitochondria. I normalized these values to account for the differences in intensities across channels. Figure 3 shows graphs showing the relative intensities of the general mitochondria marker versus Mfn1 in two cells. Figure 3A shows a distinct increase in Mfn1 intensity at the end of the mitochondrion, where mitochondria often undergo fusion events. Figure 3B illustrates an area where two mitochondria are coming together; the gap between the two is shown at the spot where the mito-mScarlet decreases to zero. At this same location, there is an increase in the Mfn1 signal between the two mitochondria, where they will undergo fusion. In comparison, two mitochondria from the Drp1 knockdown condition show no stark increase, indicating no puncta in Figure 3 C and D. Overall, the levels of Mfn1-mNG and mito-mScarlet are equal and general across these mitochondria.

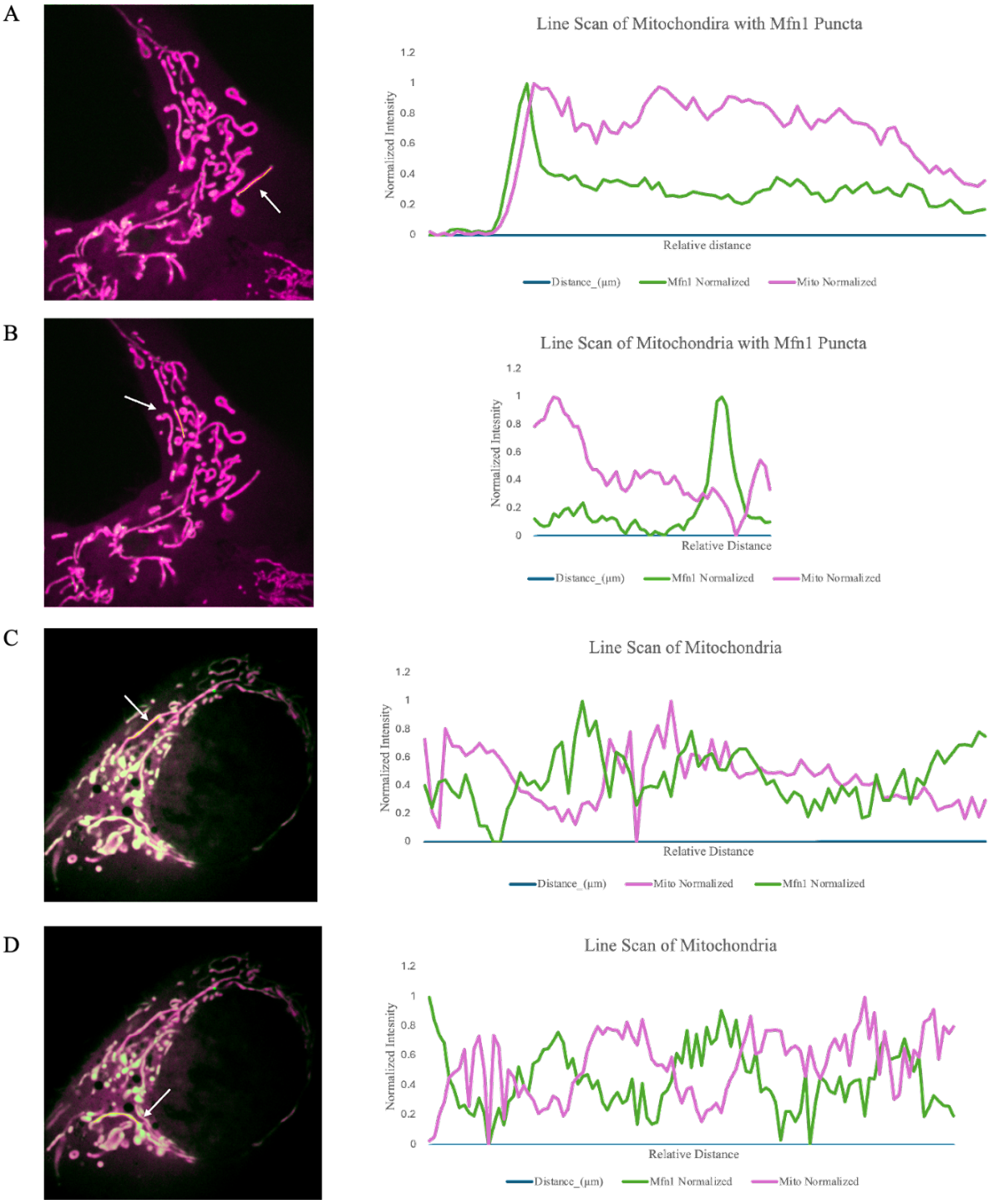


Figure 3: Line Scans of mitochondria show concentration patterns of Mfn1 in knockdown vs control conditions. A: Image showing mitochondrion selected, and corresponding line scan of comparing intensities of Mfn1 and the general mitochondria maker along length of selected mitochondrion in knockdown control cells. B: Same as A, except different mitochondrion selected. C: Same as A, except first mitochondrion selected in SiDrp1 knockdown cells. D: Same as C, except different mitochondrion selected.

To confirm that the knockdown worked, and that the phenotype difference is due to it, I did an immunoblot to show the levels of Drp1 in both samples. The two dishes were collected immediately following imaging, lysed, and run to analyze protein levels. They were blotted with an anti-Drp1 antibody and an anti-GAPDH antibody as a loading control. Figure 3A shows that there is no band for Drp1 in the knockdown cells while there is for the control cells, which indicates that this was a complete knockdown, suggesting that the phenotype results are due to the lack of Drp1. Figure 3B quantifies the intensities of the Drp1 bands controlled for any difference in loading controls. Compared to the control lane's Drp1 band, there is essentially no signal in the knockdown lane, which further indicates that the knockdown was complete and all Drp1 was suppressed.

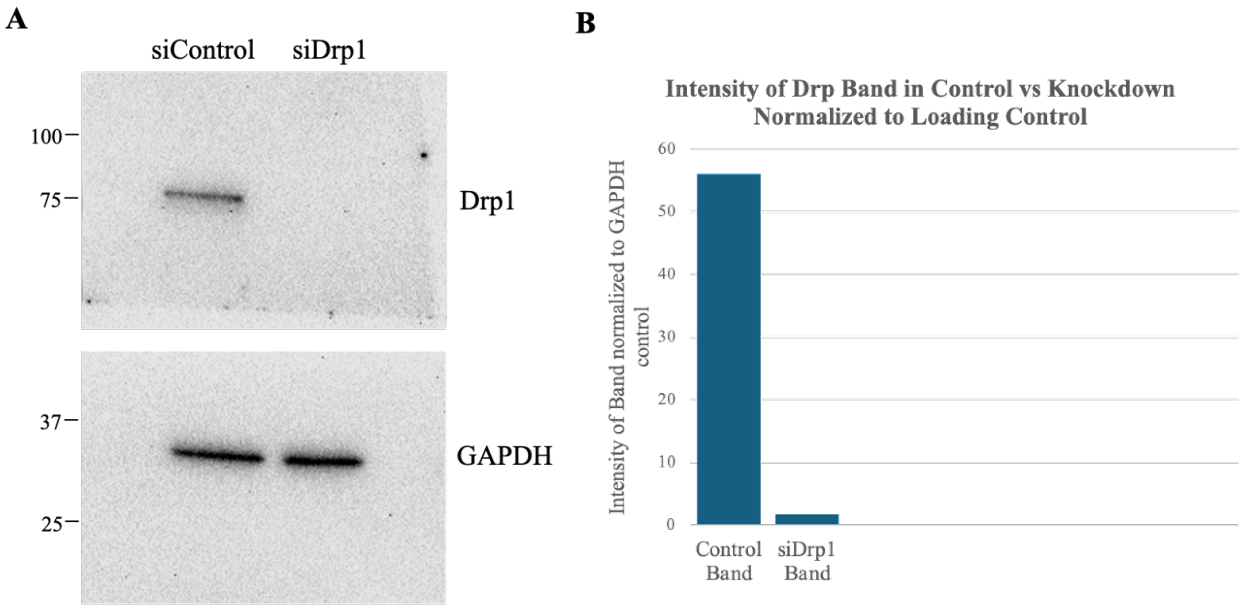


Figure 4: Presence of Drp1 depleted after knockdown. After knockdown, cells were lysed with SDS and collected for immunoblot. A: immunoblot of Drp1 knockdown and scramble control. Blotted against GAPDH as loading control and Drp1 to shown presence of protein. B: Graph quantifying band intensities.

One possibility for a difference in localization phenotype would be that there is a difference in the amount of protein. Additionally, if Drp1 is required to recruit Mfn1, there is potential for the loss of Mfn1 levels in the absence of Drp1 if it triggers a cellular process to suppress it. To test this, I performed an immunoblot against Mfn1 in both control and knockdown cells across three different experiments to measure the levels of endogenous Mfn1 and tagged Mfn1. The blot was also tested against GAPDH as a loading control. By comparing the loading controls, trial one had more cell content than either trial two or three. However, across all three trials, there is evidence of less Mfn1 in the Drp1 deficient cells (Figure 5A). To confirm these differences in Mfn1 levels, I quantified the intensities of the lanes and controlled for both background levels and differences between the two samples within one trial. Figure 5B shows that once normalized, all three trials show a decrease in the amount of endogenous Mfn1 in the knockdown cells compared to the control samples.

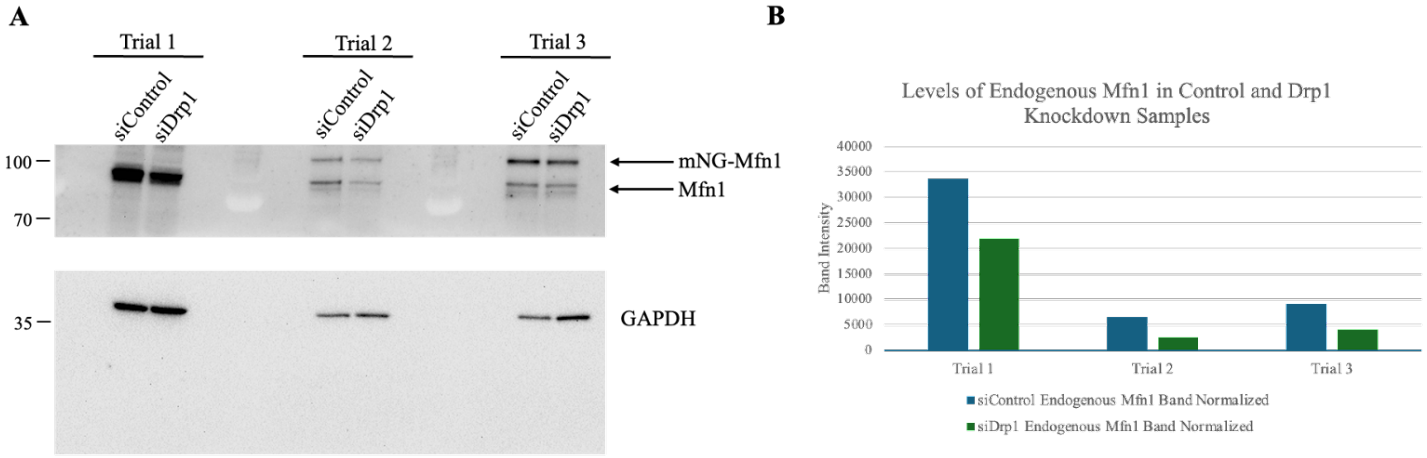


Figure 5: Endogenous Mfn1 levels decrease in Drp1 depleted cells. A: Immunoblot showing Mfn1 levels in control cells vs Drp1 knockdown cells. GAPDH shown in lower panel as loading control. B: Graph showing intensity of band to show amount of endogenous Mfn1 in both samples. Normalized by subtracting by value and averaging to differences in loading control.

Discussion

The goal of this study was to further our understanding of how the proteins involved with mitochondrial dynamics interact with each other. The general process of these events is relatively known, but there are still many gaps in our understanding. It is known that the ER regulates both the fission and fusion sites of mitochondria at the same nodes on the ER (Abrisch et al., 2020). ER proteins such as ABHD16A and mitochondrial proteins such as Drp1, Mfn1, and Opa1 are all necessary for the event to occur (Nguyen & Voeltz, 2022). This study aimed to build our understanding of how these proteins interact with each other so that we can further understand how they affect the pathway when one loses its function.

This study suggests that Drp1 may have some kind of interaction with Mfn1. When Drp1 was successfully knocked down using siRNA, there was an effect on mitochondrial morphology and Mfn1 localization (Figure 2). Overall, the mitochondria are elongated compared to the control cells, which is reasonable due to Drp1 being involved in fission, so when it's not present, they cannot split. Additionally, the cells look generally unhealthy because there are fewer dynamic events, which indicate cell health (Figure 2). Mfn1 localization is also vastly different between the knockdown and control cells. In the control cells, the Mfn1 localized as it is described in the literature, generally to the mitochondria, with bright puncta usually found at the end of a mitochondrion where fusion events often occur (Figure 3). In comparison, in cells in which Drp1 was knocked down, Mfn1 still localizes to mitochondria, but far fewer puncta were observed in these cells. The difference in puncta between the two conditions is shown to be statistically significant (Figure 2C). This indicates that even though only the fission protein was knocked down, the fusion protein still could not localize to nodes.

These results from the siRNA knockdown experiment seem to support the hypothesis that Drp1 is required to recruit Mfn1. While more experiments must be done to confirm this interaction, there are multiple potential explanations for the nature of this effect. One of these is that Drp1-associated constriction is necessary for the mitochondria sites to be defined, which allows Mfn1 to be recruited. This could also be explained by if there is a direct interaction, where Drp1 physically recruits Mfn1 to nodes. In this case, Mfn1 does not leave its general mitochondrial localized state without Drp1.

Further, it seems that Drp1 being knocked down may affect the amount of Mfn1 produced in cells. Across three experiments, the levels of Mfn1 in cells where Drp1 was depleted were significantly less than in control cells. Interestingly, when the tagged Mfn1 is compared, there also appears to be less in the knockdown cells even though consistent levels of plasmid were transfected in each (Figure 5). One explanation for this would be if Drp1 is depleted in cells, it is triggering something to degrade existing Mfn1 because more elongated mitochondria mean it is less essential to the cell's health. Additionally, if Drp1 depletion causes Mfn1 to become nonfunctional, the cell will not prioritize its production.

This study ultimately shows a potential relationship between Mfn1 and Drp1 that has not been previously described. Mitochondrial fission and fusion have been previously studied as separate events. Still, due to the proteins involved in both processes localizing to the same nodes, there could be an interaction connecting the two. Mfn1 can no longer localize to these locations and is present in lower amounts when Drp1 is knocked down in U2OS cells. More must be done to characterize the interaction between these proteins, but this study provides a base for future research by defining an impact on fusion protein localization when fission events are blocked.

Future Directions

The siRNA knockdown of Drp1 proved to significantly alter the localization of Mfn1, which indicates a relationship between the two proteins. This was shown over two replicates and twenty cells analyzed. Ideally, experiments would be analyzed and shown in at least a triplicate so more replicates should be performed to increase confidence. This would allow for more cells to be analyzed, and a larger sample size would increase the level of confidence in the average number of puncta in both conditions. Additionally, more cells could be imaged in each condition during the replicates to further increase the number of cells analyzed.

Additionally, in a siRNA knockdown experiment, it is necessary to perform a rescue experiment to conclude that the results are due to exclusively knocking down the protein. By introducing a plasmid of Drp1 that has been mutated to be resistant to the siRNA, it will be able to restore the function of Drp1. The phenotype of this control can be compared to the knockdown and the scramble siRNA to prove that the results can be attributed to specifically Drp1 being knocked down. If the rescue did not restore the cells to the wild type, then there is a potential side effect of the siRNA that should be considered. This experiment would add validity to the study results shown here and allow for more follow-up.

This study raises various questions about the mechanism of mitochondrial fission and fusion that can further be studied. One area to study is the physical interaction between Mfn1 and Drp1. To prove whether the two proteins directly interact, a co-immunoprecipitation experiment could be done to identify if they form a complex. If the two have a strong bond, then targeting one of the proteins with a specific antibody would also pull off the interacting protein. An immunoblot could be used to show the presence of both proteins in a complex. This technique would work well if the two bind tightly, but the nature of fission and fusion events happening

quickly and then Drp1 and Mfn1 puncta dissipating suggests that the interaction would likely not be strong enough to bind through a co-IP experiment. In this case of transient interaction, a crosslinking experiment could be designed to stabilize the interaction. In this approach, crosslinkers are added to the cells and freeze transient interactions. Then, it can be followed up with a co-immunoprecipitation to isolate the complex. These experiments would provide insight into how the two proteins interact, and if these experiments suggest they do so directly, adds a new step into the mechanistic pathway of dynamic events. If they don't interact directly, there can be more investigation into how Drp1 affects the localization of Mfn1, which suggests the possibility of more proteins involved in this process.

Another follow-up to this study would be to identify other necessary proteins. As mentioned above, there may be other proteins involved in how Drp1 recruits Mfn1. Even in the case of direct interactions, there are additional questions concerning other steps of fission and fusion events. Namely, the proteins involved in tethering the mitochondria to the ER are still largely unknown. To study this, a TurboID experiment could be done to identify proteins in the same vicinity as other fission and fusion proteins. For this, specific proteins that strongly localize to nodes could be manipulated to create a construct in which they are attached to a TurboID protein. Mid49 and a mutated form of ABHD16A that targets it to the mitochondria are both targets that specifically localize nodes without a lot of noise throughout the cell. Additional constructs that localize to the general mitochondria but not specifically nodes can be created to be a control for general mitochondrial proteins that might interact with the nodes just by chance. When added, biotin will bind to the TurboID protein, thus biotinylate the target protein and anything that interacts with it. This solution can be purified to isolate the proteins attached to

biotin and can be identified with mass spectrometry. The identified proteins can be screened for potential involvement and further studied to understand their role in the process.

Materials and Methods

Cell Culture and Transfection

U2OS (ATCC, HTB-96) cells were used for all experiments. They were grown in McCoy's 5A Medium (with an addition of 5mL 100X penicillin/streptomycin and 50mL of fetal bovine serum). Cells were grown in 5mL flasks, until reaching 80% confluency, then split by washing with PBS, and treating with 1mL 0.25% trypsin + EDTA for 5 minutes. Cells were then divided into new flasks or imaging dishes based on use.

For imaging, cells were plated in 2mL imaging dishes that had been treated with 1X Fibronectin the day before transfection. Transfections were 500µL containing P3000/Lipo3000 lipofection transfection agents, Opti-MEM media, and the plasmids to be imaged. Transfection was left on cells in 37° incubator for 5 hours, then washed with complete McCoy's 5A Medium and left to incubate overnight. Imaging experiments were performed with IMAGING MEDIA (what is it) at 37ºC.

For imaging experiments, the following amount of DNA was transfected: 125ng mNG-Mfn1, 100ng mito-mScarlet, 5 µL of 10 µM scramble siRNA, and 5 µL of 10 µM siRNA targeting Drp1.

Western Blot

SiRNA knockdown samples were separated on SDS-PAGE (percentages) gels at 200V for 35 minutes. Samples were transferred onto PVDF membranes for 1 hour at 4ºC at 50V. The membrane was blocked in 5% nonfat milk in TBST for 30 minutes and then moved into primary antibodies overnight at 4ºC. The membranes were then washed 4 times in TBST in 5-minute intervals and transferred into secondary antibody solutions for 1 hour. Membranes were washed

4 times with TBST in 5 minute intervals and imaged with SuperSignal Pico Reagent (ThermoFischer, 34580) or SuperSignal West Femto Reagent (ThermoFischer, 34096). The primary antibodies used: α-GAPDH (Sigma, G9545), α-Dnm1 (Invitrogen, PA1-16987; 1:1000), α-Mfn1 (Proteintech, 13798-1-AP; 1:2000). The secondary antibodies used: Goat α-Rabbit (Sigma, A6154, 1:5000), and Goat α-Mouse (Sigma, A4416, 1:3000).

Western blot lanes were quantified using FIJI. The blot was inverted, and a box was drawn around the largest band. That box was maintained around every band to be measured. The same box was also used to measure a background measurement in each lane. The quality of the loads was measured by creating a ratio of the difference between the loading controls. The intensity was calculated by subtracting the background signal and then multiplying the lower signal by the ratio determined by the difference in loading control intensities. Calculations and graphs were made in Excel.

Construct Creation

mNG-Mfn1, Mito-mScarlet, and Mito-BFP have all been previously used and described. SiRNA targeting Drp1 (Dharmacon L-012092-00-0005) was composed of 4 target sequences:

GGAGCCAGCUAGAUAUUAA;

CAUCAGAGAUUGUUUACCA;

CGUAAAAGGUUGCCUGUUA;

GUGAACCCGUGGAUGAUAA.

siRNA control was made up of a scrambled non targeting siRNA that would not suppress any proteins.

Microscopy and Image Analysis

All images were taken on a Nikon eclipse Ti2 inverted microscope equipped with a 100× 1.45-NA Plan Apo objective, Yokagowa CSU-X1 spinning disk confocal scanner, an Andor iXon 897 electron-multiplying charge-coupled device camera, and OBIS LX/LS lasers (405/488/561/640 nm). Each movie was made up of 21 images taken in 5 second time intervals. The chamber was held at 37ºC throughout the duration of imaging.

Images were processed in FIJI. Line scans were taken by drawing a freehand line down the length of a mitochondrion in the mito-mScarlet channel. FIJI then calculated intensities of both channels along this line. Excel was used to normalize data and plot it onto graphs.

Mfn1 puncta were analyzed in twenty cells across two separate siRNA knockdown experiments. Movies analyzed were 21 images in 5 second time intervals. Puncta were defined as spots of mNeonGreen signal above set threshold. All thresholds were manually set in Matlab. The average number of puncta was calculated in Excel using "average" function. P-value was calculated between control and knockdown in Excel using the function "T.TEST".

siRNA knockdown

Cells were plated in two wells of a six-well plate. The next day, one well was treated with 50pmol siRNA against Drp1(Horizon/Dharmafect, L-012092-00-0005), Dharmafect reagent 1 (Horizon/Dharmafect, T-2001-02), and serum and antibiotic-free McCoy's 5A medium. The other well was treated with 50pmol scramble siRNA control, Dharmafect reagent 1, and serum and antibiotic-free McCoy's 5A medium. Both transfections were left for 6 hours at 37ºC and then returned to full media at 37º overnight. The next day, both wells were split into 2mL fibronectin-treated imaging dishes. On day 4, cells were transfected with 50pmol of either the Drp1 specific siRNA or the scramble siRNA, 150ng mito-mScarlet, 125ng mNG-Mfn1, Lipofectamine 3000

reagent, and Opti-mem. The plates were left at 37ºC for 5 hours, then returned to full media overnight. On day 5, cells were imaged using the Microscopy techniques above. The cells were harvested for western blot by washing off media with PBS and treating with Trypsin for 10 minutes at 37ºC. Cells were spun down, washed with PBS, and then resuspended in 1X SDS sample buffer containing 5% β-mercaptoethanol. Samples were boiled at 95ºC for 5 minutes.

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