THE ROLE OF DMIRO IN THE DISTRIBUTION AND HEALTH OF MITOCHONDRIA

BY

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Abstract

In neurons, regions of high energy demand, like synapses, can be far away from the cell body. Therefore, neurons require efficient mitochondrial transport for maintaining synaptic function and preventing degeneration. Miro is a protein that has been found to be necessary for proper distribution of mitochondria into axons and dendrites of neurons. Miro has an N-terminal GTPase domain (G1), a C-terminal GTPase domain (G2), two calcium-binding EF-hand domains, a variable domain of unknown function, and a transmembrane domain that anchors the protein to the outer mitochondrial membrane. This project examines two main questions concerning the role of *Drosophila* Miro (dMiro) for mitochondrial transport. First, it examines whether the potentially constitutively active mutations A20V in the G1 domain and K455V in the G2 domain of dMiro affect kinetics of mitochondrial transport. Second, it examines the potential role of the variable domain of dMiro for mitochondrial transport, function, and morphology. I found that the A20V mutation in the G1 domain had no significant effects on the kinetics of mitochondrial transport, even though loss-of-function mutations of the domain abolished the distribution of mitochondria into axons and dendrites. Furthermore, the K455V in the G2 domain had no effect on transport, and did not modulate the function of the G1 domain. Finally, I found that the variable domain of dMiro confers different functional characteristics to the three different isoforms of dMiro. Specifically, I found that the medium dMiro isoform interacts differently with the mitochondrial fusion protein Marf.

Introduction

Cells are constantly undergoing different processes that need energy. They may be transcribing proteins, growing, or even digesting substances. To do all this, there needs to be a way for them to regulate where energy is created and it needs to align with where energy is being used. Cells have organelles called mitochondria that synthesize ATP, which is the main source of energy for a cell. In neurons, however, getting mitochondria to the places where energy is used can be a problem, since axons and dendrites can extend large distances from the cell body.

Transport of mitochondria in neurons is largely achieved through the use of motors and tracks provided by microtubules (MT) or actin. Microtubules are polar, a property imparted by the manner of their polymerization; the alpha- and beta-tubulin subunits are added to a growing MT on the plus-end, while the minus-end remains anchored. The polarity of MT is critical for motors using MT as tracks to transport cargo. Most motors of the kinesin family transport cargo towards the plus-end of the MT while motors of the dynein family transport cargo toward the minus-end. Thus, directionality of MT-based transport can be achieved by controlling which motor is active at any given time (Franker & Hoogenraad, 2013; Rolls et al., 2007).

In axons of neurons, MTs are organized with their plus-ends pointing toward the axon terminal. In contrast, dendrites of mammalian neurons exhibit a mixed organization of MT polarity. However, neurons of *Drosophila* (used in this project) exhibit a MT organization of uniform polarity with plus-ends pointing towards the cell body. Thus, I could easily study kinesin activity by looking at axons of motor neurons or dynein activity by looking at dendrites of sensory neurons (Franker & Hoogenraad, 2013; Rolls et al., 2007).

Mitochondria in cells are constantly undergoing fission and fusion processes. Repetitive cycles of fusion and fission serve a quality control mechanism sorting dysfunctional mitochondrial proteins into one of the two daughter mitochondria, which is then targeted for degradation (reviewed in Youle & van der Bliek, 2012). In *Drosophila*, mitochondrial fusion is mediated by Marf, the homolog of mammalian Mitofusin. Additionally, drp1 mediates mitochondrial fission (Chan, 2012).

The central subject of my thesis is the *Drosophila* homolog of the atypical mitochondrial GTPase Miro (dMiro). It has two GTPase domains, two calcium-binding EF-hand domains, a variable domain, and it is anchored to the cytosolic side of the outer mitochondrial membrane by a transmembrane domain (Fig. 1). At the variable domain, dMiro can be alternatively spliced

into three different isoforms—a long, medium, and short form (Fig. 2)(St. Pierre SE, 2014). Those isoforms each have differential effects on the health of mitochondria which need to be studied further.

Fig 1. The structure of Miro protein. Miro consists of the N-terminal GTPase domain (G1), two calcium-binding EF-hand domains, a C-terminal GTPase domain (G2), and a variable domain. It is anchored on the cytosolic side of the outer mitochondrial membrane by a transmembrane helix.

Evidence for the importance of dMiro for the transport of mitochondria came from imaging studies of dMiro in *Drosophila* motor axons and neuromuscular junctions (NMJs). Very few mitochondria were found in the axon or at the NMJ of dMiro null mutants (gene deletion mutation); instead, mitochondria accumulated in the cell body. Not surprisingly, viability of dMiro null mutants was seriously diminished to the point that the mutant larvae never reach adulthood. The lethality of dMiro null mutants can be rescued by the neuronal expression of normal dMiro, suggesting that only MT-based transport of mitochondria in neurons is critical for survival. Therefore, problems with dMiro deletion are strictly neural and presynaptic in nature (Guo et al., 2005).

Fig 2. Miro has three isoforms of differing lengths. These isoforms and the variable domain at which they are formed are conserved across animals ranging from Drosophila to mice to humans.

dMiro has a well-established interaction with kinesin. It acts as an anchoring protein for the adaptor-motor protein complex consisting of the adaptor Milton, which connects kinesin motors to Miro and by extension mitochondria (Glater et al., 2006). When this complex is disrupted by introducing kinesin-binding fragments, transport of mitochondria to neuronal processes is diminished (MacAskill et al., 2009). Miro was also shown to be involved in

mitochondrial trafficking using constitutively active or dominant negative mutations on its GTPase domains in cultured cells. In both cases, the mutant Miro caused abnormal clustering of mitochondria in the cell body (Fransson et al., 2006).

In healthy neurons, mitochondria must be distributed into both the dendrites and the axons despite opposite polarities. This is generally solved by regulating which motor is active. It has been shown that kinesin is responsible for targeting mitochondria into the axons while the dynein/dynactin complex is responsible for targeting them into the dendrites. Furthermore, the different Milton isoforms seem to dictate which motor is active on a mitochondrion at any given time. One isoform of Milton (TRAK1 in humans) associates with kinesin and the dynein/dynactin complex while a different isoform of Milton (TRAK2 in humans) associates with the dynein/dynactin complex. This differential affinity for the two motors is likely due to the different conformations of the Milton isoforms (van Spronsen et al., 2013).

Miro also seems to have a role in endoplasmic reticulum (ER) interactions. In eukaryotic cells, mitochondria and the ER form the endoplasmic reticulum-mitochondria encounter structure (ERMES). It consists of four proteins and is important for mitochondrial maintenance (Stroud et al., 2011). Miro's homolog in yeast serves two main roles and they are related to this ERMES. First, it regulates lipid exchange and synthesis through signaling at G2. Second, it facilitates undocking of the mitochondria from the ERMES through signaling at G1 (Kornmann et al., 2011).

This project addressed dMiro's role in transport by looking at the role of three different domains. In the first part of the project, the roles of G1 and G2 were examined by use of various gain-of-function and loss-of-function mutations. This showed important implications for G1 in distribution of mitochondria, but G2 likely serves some other function. The second part of the project looked at the variable domain and its possible implications for the fusion and fission of mitochondria.

Methods

Animal Model

The experiment was done using 3rd instar climbing *Drosophila* larvae. Each larva was systematically dissected, fixed, washed, and incubated in antibody. After dissection, they were fixed in 4% Paraformaldehyde (PFA) in Phosphate-buffered Saline (PBS) of pH 7.3. Then they were washed twice in PBS followed by three washes in PBS supplemented with 0.2% Triton-X detergent (PBS-T) to permeabilize them. After the third wash in PBS-T, they were incubated in Anti-GFP AlexFluor488 and Anti-HRP Cy3 antibodies. A mitochondria-targeted GFP manipulation allowed the AlexFluor488 to visualize mitochondria while the Cy3 would visualize nerves by binding to endogenous molecules in the nerve.

Dissection

Larvae were allowed to mature until they were 3rd instar at which point they were selected and placed on a petri dish with Sylgard resin. Using metal pins, they were pinned dorsal side up and subsequently cut open. Their body walls were then pinned such that their insides were visible. The guts were removed in a manner that revealed the ventral nerve cord and its protruding motor neurons without damaging them.

Mitochondria Tracking

A region of a nerve proximal to the ventral nerve cord was initially photobleached for 180 seconds. Images of that region were then taken at a rate of one image per 1.006 seconds for 200 frames. This allowed easy detection of motile mitochondria entering the previously photobleached region. For each of the 200 frames, the same location on a given mitochondrion was marked. Using ImageJ tracking software, motile mitochondrial kinetics was calculated.

Imaging Marf

Imaging was done using an Olympus FV300 laser confocal microscope. The lasers used were Argon488 (to visualize the AlexFluor488) and HeNe543 (to visualize the Cy3). Dissected larvae were placed under the laser and imaged using an Olympus LUMPlanFl 60x water lens.

Marf Image Analysis

The second part of the project involved looking at the different morphological effects of Marf on each isoform of dMiro. Images were therefore analyzed using ImageJ software and looked specifically at the lengths of the mitochondria since that is the morphological attribute that Marf affects. For every distinguishable mitochondrion, a line was drawn from the beginning of it to the end. Those lines were then measured and their lengths compared.

Results

Analysis of dMiro's GTPase domains

To examine the interaction of the two GTPase domains, videos of mitochondrial transport were taken and tracked as per established protocols (Russo et al., 2009; Louie et al., 2008). Using this method, we imaged mutants with various loss-of-function mutations and gain-of-function mutations in order to better understand the GTPase domains. These genes were driven by the UAS-Gal4 system with an OK6 or elav-c155 driver. When examining dendrites, the driver 21-7 was used to express the genes in dendrites. T25N and T460N were loss-of-function mutations for G1 and G2, respectively. A20V and K455V were gain-of-function mutations for G1 and G2, respectively. Using these mutations, we could look at what role each GTPase played individually. Additionally, we examined double mutants in which G1 was either turned off (T25N) or turned constitutively active (A20V) with G2 either turned off (T460N) or constitutively active (K455V) in order to see if the GTPases modulated each other.

We found that in T25N double mutants, mitochondria did not distribute into axons or dendrites, regardless of the status of the second GTPase domain. Instead, they accumulated in the soma of motor neurons. As a result, it is clear G1 is absolutely necessary for proper transport function.

Furthermore, the N-terminal GTPase negatively affects dynein activity in retrograde transport. While the knockout severely decreases distribution of mitochondria in distal areas like the synapse or NMJ, there were still some mitochondria that kinetic data could be taken from. Retrograde velocity and distance traveled by mitochondria were reduced from normal levels, but not quite as severe as dMiro null levels.

At this point, it was important to address the problem that impaired retrograde transportation may be due to the initial anterograde transportation problem; if there are not mitochondria in distal regions in the first place, the data suggesting impaired retrograde transport might be misleading. However, by imaging the distribution of mitochondria in the dendrites of sensory neurons, it was found that mitochondria failed to distribute into the dendrites and the dendritic trees degenerated from normal sizes. This suggests that the N-terminal GTPase is indeed necessary for dynein transport and the deficits in retrograde transport in motor neurons was not just a consequence of impaired kinesin activity.

In contrast, the C-terminal GTPase is not critical for normal mitochondrial transport and distribution. Neither loss-of-function nor gain-of-function manipulations of the C-terminal GTPase resulted in reduced viability of *Drosophila*. Furthermore, statistically normal distribution of mitochondria in axons and NMJs were observed in these mutations.

After determining whether each GTPase was individually necessary for mitochondrial transport, there was still a question of whether or not the C-terminal GTPase modulated the N-terminal GTPase. In the A20V mutants, we found that the mitochondrial kinetics were unaffected by either the K455V or the T460N mutations, indicating that the transport functions of the first GTPase domain are not modulated by the second. We found that for anterograde transport, the average mitochondrial net-velocity remained the same at 8.784 μm/min ($p>0.05$), and that time spent on plus-end trips was also unaffected from single mutation A20V mutants. Similarly, distances of kinesin-driven runs (defined as a distance traveled in a single direction without stopping) and trips (defined as multiple runs in the same direction which were interrupted by stops) were unaffected ($p>0.05$)(Fig. 3).

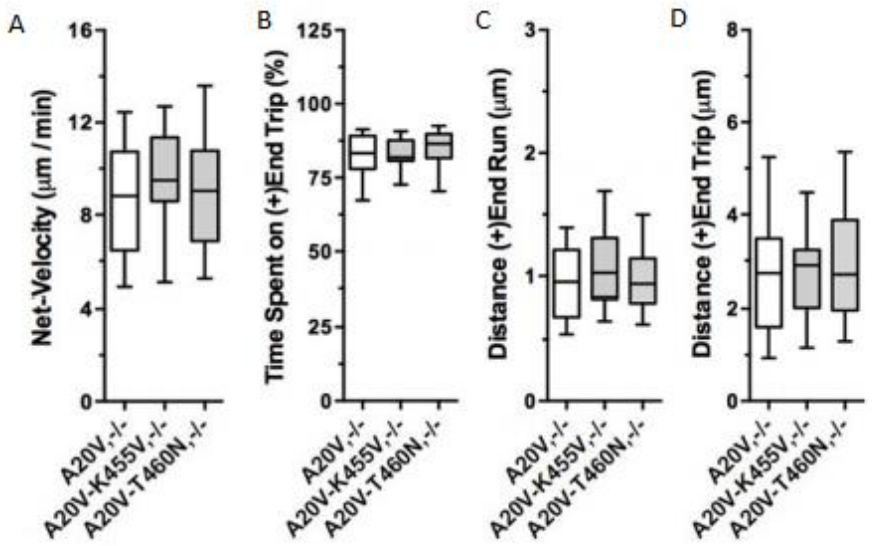

Fig 3. Adapted from Babic et al, 2015. Kinetics of A20V mutants and double mutants. Neither the net-velocity (A), time spent on (+) end trips (B), distance travelled in (+) end runs (C), nor distance travelled in (+) end trips (D) change significantly in A20V mutant animals regardless of how G2 domain was manipulated.

Analysis of dMiro's variable domain

In the second part of my work, focus was moved from transport functions of the GTPase domains to possible roles of dMiro's variable domain on the health of mitochondria. Because expression of the different isoforms in a null background results in changes in mitochondrial morphology and length, it was logical to see if the isoforms differentially facilitated a process that would influence morphology and length. One option is fusion or fission. By co-overexpressing the isoforms with Marf or drp1, we could look at how each isoform may influence fusion or fission. The UAS-Gal4 system was used again with the OK6 driver. Overexpression of a gene will cause an exaggerated phenotype for that gene, which allows us to better understand the effect of Marf and drp1 on the isoforms. Results indicated overexpression of Marf increased the length of mitochondria in axons of wildtype animals, as well as in axons of animals co-overexpressing the long and short isoforms of dMiro (Table 1). However, the increased length phenotype was suppressed in animals co-expressing the medium isoform of dMiro (Fig. 4). This phenotype can be observed visually in Fig. 5 as well.

Genotype	Average Length (µm)
Control	1.328
Marf OE	8.099
dMiro L OE	2.829
dMiro L OE, Marf OE	6.246
dMiro M OE	1.74
dMiro M OE, Marf OE	1.891
dMiro S OE	4.366
dMiro S OE, Marf OE	15.28

Table 1. Average mitochondrial lengths for Marf animals.

Genotype	Average Length (µm)
Control	1.328
drp1 OE	2.33
dMiro L OE	2.829
dMiro L OE, drp1 OE	2.787
dMiro M OE	1.74
dMiro M OE, drp1 OE	1.681
dMiro S OE	4.366
dMiro S OE, drp1 OE	6.421

Table 2. Average mitochondrial lengths for drp1 animals.

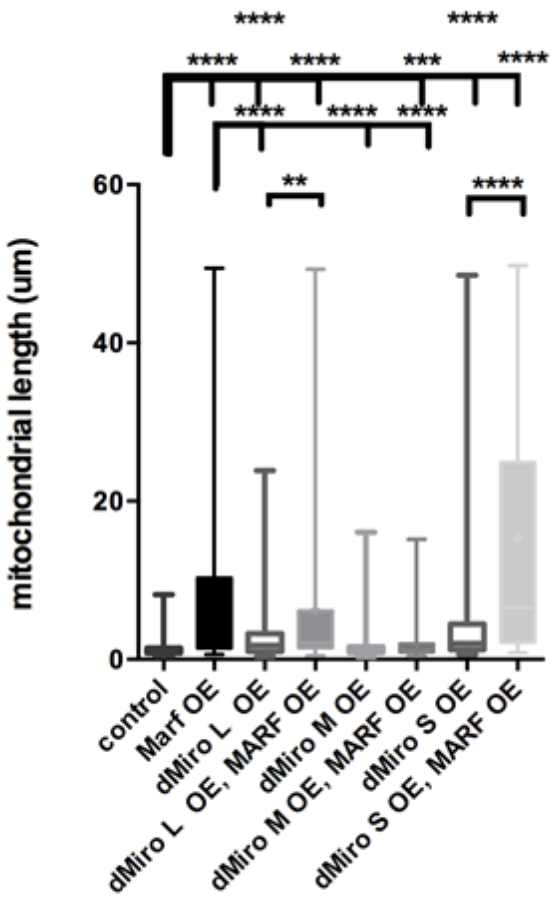

Fig 4. Effects of the addition of Marf OE. Mitochondrial length increases when Marf is overexpressed in wildtype animals, or in animals which co-overexpress dMiro-L or –S. In animals which co-overexpress dMiro-M, the mitochondrial size is unaffected by Marf OE.

The results with drp1 overexpression were problematic. The lengths actually increased, which is inconsistent with overexpression of a fission protein and indicative of a contamination of the line. Table 2 shows average lengths of mitochondria in each genetic line. As a result, the data was disregarded.

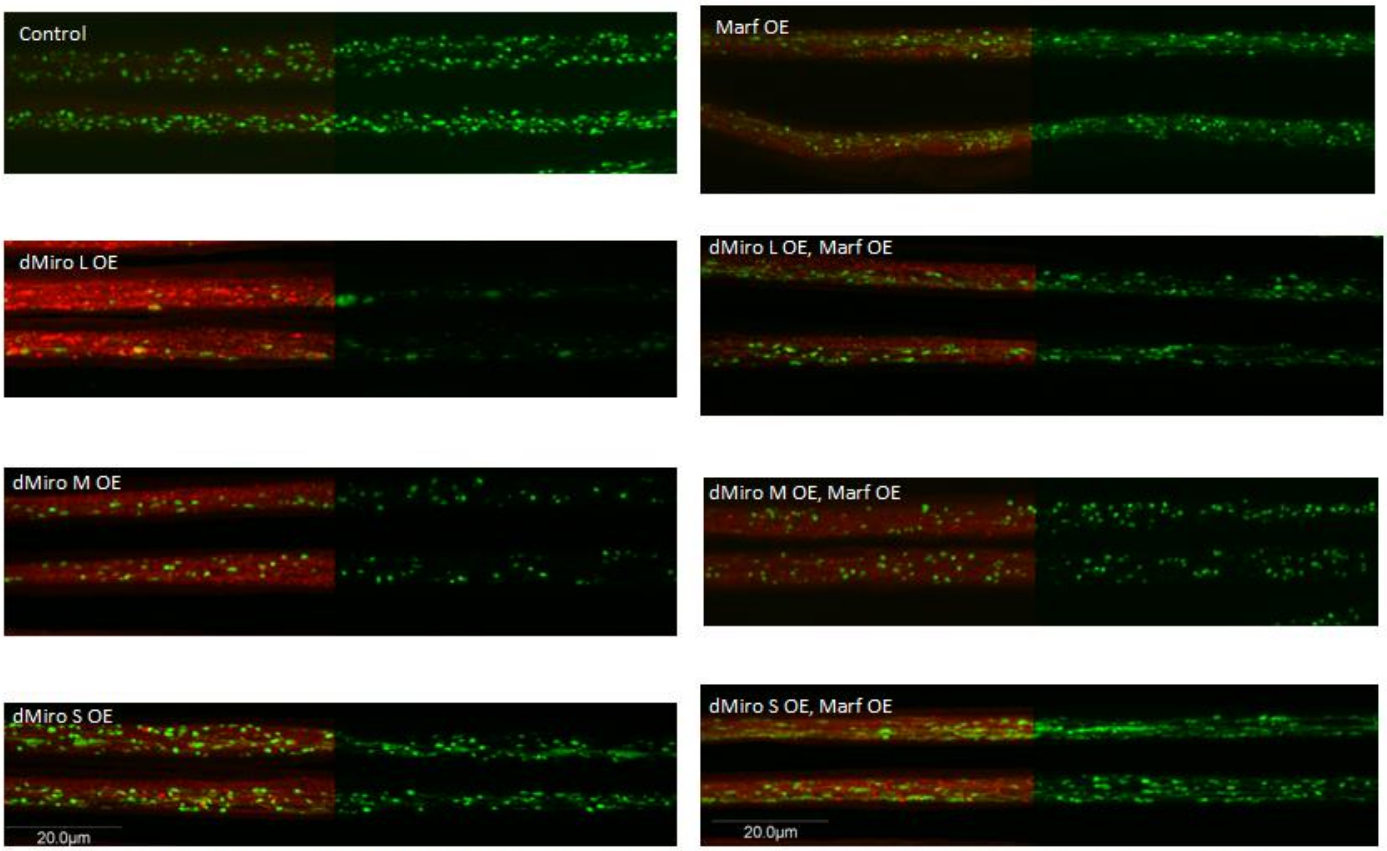

Fig 5. Effects of dMiro isoform overexpression on mitochondrial density and morphology. The left column shows the differences in transport rescue for each isoform in a *dmiro* null background. The right column shows the co-overexpressions where the differential interaction with Marf can be seen.

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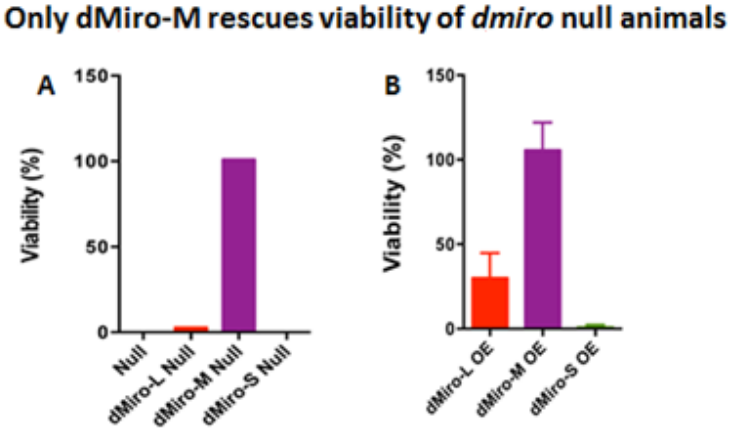

Fig 6. Viability numbers for dMiro isoform rescues. (A) Percent survival in animals expressing dMiro isoforms in *miro* null background. Only dMiro-M is capable of restoring normal viability. (B) Percent survival in wild type animals overexpressing dMiro isoforms. Both dMiro-L and –S severely reduce viability when overexpressed; OE of dMiro-M has no effect.

Discussion

Understanding specific functions of each domain of a protein is the first step to understand how it fits in to the complicated workings of the cell. This project looked at the two GTPases and the variable domain of dMiro.

Our results indicate that dMiro's N-terminal GTPase domain is absolutely required for mitochondrial transport. When dMiro is mutated to disrupt G1 function, mitochondria do not leave the cell body by using either kinesin- or dynein-driven transport. Rather, the mitochondria accumulate in cell bodies. In addition, it is evident that the C-terminal domain of Miro does not modulate the activity of the N-terminal domain because turning G2 active or inactive did not have an effect on G1 phenotypes. G2 most likely does not play any significant role in transport, either, because a loss-of-function mutation at that domain did not affect mitochondrial transport.

There are multiple possible mechanisms for how the N-terminal GTPase might facilitate transport. One possibility is that it controls how active a particular motor is by either disconnecting it from the MT or disconnecting it from the Miro/Milton complex altogether. It could also facilitate transport by physically disconnecting it from a stationary object, namely the endoplasmic reticulum (ER). This would be consistent with findings in yeast where G1 is critically important for undocking mitochondria from the ER (Kornmann et al., 2011). It is possible that this function is preserved in higher animals. However, we still need to identify other proteins that might be involved with the activator complex, or other proteins which initiate or suppress Miro's activity. We also need to understand what the C-terminal GTPase does, since it is most likely not involved directly in transport. A third topic needing further investigation is the exact nature of the gain-of-function mutations. As of now, they are "presumed" gain-of-function mutations based on similar mutations in a different system (Fransson et al, 2003). Our data instead suggests they may be neomorphic mutations, inducing an unnatural interaction or function of the dMiro protein. It would be good to gather more information about the implications of these mutations.

The isoforms of dMiro, the subject of the second part of my work, are conserved across *Drosophila*, mice, and humans. Thus, these different lengths of the protein seem to be evolutionarily important otherwise they would not be so well conserved (Fig. 2). It is clear that they do not primarily exist for mitochondrial transport purposes despite that being a main role of dMiro as a whole. This is evident when using each isoform individually as a rescue to

mitochondrial transport in a null background. While each isoform can rescue mitochondrial transport, there are differences in the morphology of the mitochondria (Fig. 5) and the viability of the animals (Fig. 6). The isoforms may still be involved in transport as a byproduct of having different roles, however. To test the possible role of isoforms in fusion (to facilitate alternate distribution of mitochondrial size), we co-overexpressed Marf with each isoform. It is clear that fusion modulation is related somehow to dMiro isoform expression because the medium isoform did not show increases in length upon introducing Marf. To better illuminate this relationship, it would be good to see the effects Marf has in the dendrites of neurons.

Unfortunately, our studies with Drp1 failed. The data showed an increase in length characteristic of fusion rather than the expected decrease in length characteristic of fission. This issue was likely due to a contamination of the fly line. Thus, an obvious direction to go to explore the fission and fusion relationship with dMiro would be to look at the effects of Drp1 using an uncontaminated line.

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