MOLECULAR MECHANISMS OF MITOCHONDRIAL TRANSPORT IN NEURONS

by

Milos Babic

A Dissertation Submitted to the Faculty of the
GRADUATE INTERDISCIPLINARY PROGRAM IN NEUROSCIENCE

In Partial Fulfillment of the Requirements
for the Degree of
DOCTOR OF PHILOSOPHY
In the Graduate College

THE UNIVERSITY OF ARIZONA

2015
As members of the Dissertation Committee, we certify that we have read the dissertation prepared by Milos Babic, titled Molecular Mechanisms of Mitochondrial Transport in Neurons and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

Date: 04/08/2015

Konrad Zinsmaier

Date: 04/08/2015

Lisa Elfring

Date: 04/08/2015

Daniela Zarnescu

Date: 04/08/2015

Julie Miller

Final approval and acceptance of this dissertation is contingent upon the candidate’s submission of the final copies of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Date: (04/08/2015)

Dissertation Director: Alan Nighorn
STATEMENT BY AUTHOR

This dissertation has been submitted in partial fulfillment of the requirements for an advanced degree at the University of Arizona and is deposited in the University Library to be made available to borrowers under rules of the Library.

Brief quotations from this dissertation are allowable without special permission, provided that accurate acknowledgment of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the Graduate College when in his or her judgment the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

SIGNED: Milos Babic
ACKNOWLEDGEMENTS

I would like to express my gratitude to my undergraduate students, who assisted with dissections and immunohistochemistry washes for many of the experiments described herein. Terra Kuhn, Benjamin Hunter, Ryan Sangston, Kathryn Cruice, Hojin Seo and Rachel Langston: I hope the things you learned while we worked together can at least partially repay you for your hard work.

I furthermore owe thanks to Andrea Wellington, the best lab manager I ever worked with. Without her able assistance, my work would have been much more difficult.

I also wish to thank my current and past committee members - Lisa Elfring, Daniela Zarnescu, Julie Miller, Gregory Dussor and Wulfila Gronenberg - for their guidance and assistance. A special thanks is due to Alan Nighorn, who advised me to transfer into the Neuroscience graduate program, and without whose advice I would not be where I am today.

Last but not least, I wish to thank Konrad Zinsmaier, for many years of his mentorship, and for his diligent assistance in preparation of this thesis.
DEDICATION

This manuscript is dedicated to my parents, Jovan and Rajka Babić, who have supported me in every possible way, through all the ups and downs of my life; and to the graduate students of Zinsmaier lab - Meaghan Torvund, Garry Russo, Elliot Imler, Lilian Patron and Sarvari Panchumarthi - who have become far more than just colleagues.
# TABLE OF CONTENTS

List of Figures................................................................................................................................................. 7

Abstract ............................................................................................................................................................. 8

Chapter 1: Introduction .................................................................................................................................. 10
  1.1 The importance of mitochondrial function in neurons.............................................................................. 10
  1.1.1 Mitochondria: an overview ................................................................................................................. 10
  1.1.2 Neurons require transport of mitochondria and a dynamic control of their structure and function..... 10
  1.2. Mechanisms which control mitochondrial structure and health ............................................................ 16
    1.2.1 Mitochondrial fission and fusion ........................................................................................................ 16
    1.2.2 Mitochondrial degradation through mitophagy ................................................................................... 21
    1.2.3 Functional significance of mitochondria-ER contact sites .................................................................. 27
  1.3. Mitochondrial GTPase Miro ..................................................................................................................... 33
    1.3.1 General overview ................................................................................................................................ 33
    1.3.2 The function of Miro (Gem1) in yeast ............................................................................................... 36
    1.3.3 The function of Miro in metazoa ........................................................................................................ 40
    1.3.4 Regulation of the metazoan Miro transport complex ......................................................................... 46
    1.3.5 Main questions addressed by this study ............................................................................................. 49

Chapter 2: The Role of Miro's N-terminal GTPase Domain in Mitochondrial Transport ........................................ 50
  2.1 Summary ..................................................................................................................................................... 50

Chapter 3: The Role of Miro’s N-terminal GTPase Domain in Mitochondrial Transport ........................................ 53
  3.1 Abstract ..................................................................................................................................................... 53
  3.2 Introduction ............................................................................................................................................... 54
  3.3 Methods ................................................................................................................................................... 57
  3.4 Results ..................................................................................................................................................... 60
  3.5 Discussion ............................................................................................................................................... 73

Chapter 4: Summary and Future Directions .................................................................................................... 76

Appendix A: Miro’s N-Terminal GTPase Domain Is Required for Transport of Mitochondria into Axons and Dendrites ............................................................................................................. 78

Appendix B: Statement of Permissions ............................................................................................................ 124

References ......................................................................................................................................................... 125
LIST OF FIGURES

Figure 1. Structure of Miro protein ......................................................................................................................... 34
Figure 2. The Function of Miro in yeast ...................................................................................................................... 38
Figure 3. Miro is required for "undocking" of mitochondria from the ER ................................................................. 39
Figure 4. The Function of Miro in metazoans ........................................................................................................... 43
Figure 5. Structure of the Drosophila milton gene ....................................................................................................... 61
Figure 6. Effects of chronic Milton-A and Milton-B overexpression on mitochondrial distribution in motor neurons .......... 63
Figure 7. Effects of acute Milton-A and Milton-B overexpression on mitochondrial distribution in motor neurons .................. 64
Figure 8. Effects of Milton-B overexpression on mitochondrial distribution in sensory neurons ........................................ 66
Figure 9. Miro and its N-terminal GTPase domain are required for Milton facilitation of transport ................................ 69
Figure 10. Milton-B OE phenotype overrides the mitochondrial accumulation at NMJs caused by dMiro OE ......................... 71
Figure A1. Transgenic expression of GTPase-mutant dMiro proteins ................................................................................ 91
Figure A2. dMiro’s N-terminal GTPase domain is required for a normal distribution of mitochondria in motor neurons ................ 94
Figure A3. Effects of mutations in dMiro’s GTPase domains on the distribution of synaptic components .......................... 97
Figure A4. dMiro’s N-terminal GTPase domain is required for normal motility of axonal mitochondria .............................. 99
Figure A5. Effects of dMiroT25N and dMiroT460N on kinesin-driven motility during anterograde axonal transport of mitochondria .......................................................... 102
Figure A6. Effects of dMiroT25N and dMiroT460N on dynein-driven motility during retrograde axonal transport of mitochondria .................. 105
Figure A7. Comparison of dMiroT25N- and dMiroT460N-induced phenotypes in the absence and presence of endogenous dMiro .................................. 106
Figure A8. Effects of dMiroT25N on the distribution of mitochondria in dendrites of sensory neurons .......................... 108
Figure A9. Effects of dMiro and dMiroT25N overexpression on the distribution of mitochondria in dendrites of sensory neurons ..................................................... 110
Figure A10. Effects of dMiroA20V and dMiroK455V on kinesin-driven motility during anterograde axonal transport of mitochondria .................................................. 114
Figure A11. Effects of dMiro GTPase double mutant proteins on the distribution and transport of mitochondria in axons ................ 116
ABSTRACT

Dynamic mitochondrial transport into axons and dendrites of neuronal cells is critical for sustaining neuronal excitability, synaptic transmission, and cell survival. Failure of mitochondrial transport is the direct cause of some neurodegenerative diseases, and an aggravating factor for many others. Mitochondrial transport regulation involves many proteins; factoring prominently among them are the atypical mitochondrial GTPase Miro and the Milton/TRAK adaptor proteins, which link microtubule (MT) motors to mitochondria. Motors of the kinesin family mediate mitochondrial transport towards the plus ends of microtubules, while motors of the dynein family mediate mitochondrial transport towards the minus ends. Selective use of these motors determines the ultimate subcellular distribution of mitochondria, but the underlying control mechanisms remain poorly understood.

*Drosophila* Miro (dMiro) is required for kinesin-driven transport of mitochondria, but its role in dynein-driven transport remains controversial. In Chapter 2 of this study, I show that dMiro is also required for the dynein-driven transport of mitochondria. In addition, we used the loss-of-function mutations dMiroT25N and dMiroT460N to analyze the function of dMiro's N- and C-terminal GTPase domains, respectively. We show that dMiroT25N causes lethality and impairs mitochondrial distribution and transport in a manner indistinguishable from *dmiro* null mutants. Our analysis suggests that both kinesin- and dynein-driven mitochondrial transport require the activity of Miro's N-terminal GTPase domain, which likely controls the transition from a stationary to a motile state irrespective of the transport direction. dMiroT460N reduced only dynein motility during retrograde axonal transport but had no effect on distribution of mitochondria in neurons, indicating that the C-terminal GTPase domain of Miro most likely has only a small modulatory role on transport. Furthermore, we show that commonly used
substitutions in Miro's GTPase domains, based on the constitutively active Ras-G12V mutation, appear to cause neomorphic phenotypic effects which are probably unrelated to the normal function of the protein.

In mammalian neurons, kinesin and dynein motors are linked to mitochondria via a Miro complex with the adapter proteins TRAK1 and TRAK2, respectively. Differential linkage of TRAK-motor complexes provides a mechanism for determining the direction of transport and controlling mitochondrial distributions within the cell. Drosophila has only one TRAK gene homolog, Milton, which expresses several protein isoform. Milton has been previously been shown to facilitate mitochondrial transport by binding to kinesin and dMiro, a role analogous to TRAK1. However, the question whether Milton might be able mediate dynein-based transport in a manner similar to TRAK2 has remained unknown. In Chapter 3 of this study, I show that protein isoforms A and B of Milton, generated through alternative mRNA splicing, facilitate differential motor activities analogous to mammalian TRAKs. Specifically, overexpression (OE) of Milton-A caused a mitochondrial redistribution and accumulation at axon terminals, which requires kinesin-driven MT plus end directed transport; while OE of Milton-B caused a redistribution of axonal mitochondria into the soma, which requires dynein-driven MT minus end directed transport. I further show that Milton-motor complex binding to mitochondria requires Miro exclusively, and that transport with either of the motor complexes absolutely requires the activity of Miro's N-terminal GTPase domain.

Together, these results suggest that Miro controls the transition of mitochondria from a stationary to a motile phase. Thereafter the direction of transport is likely determined by an alternative binding of opposing Milton/TRAK-motor complexes to Miro, a process which appears to be regulated by a Miro-independent mechanism.
CHAPTER 1: INTRODUCTION

1.1 The importance of mitochondrial function in neurons

1.1.1 Mitochondria: an overview

Mitochondria are a near-universal feature of eukaryotes, intimately linked with the very origin of complex life on Earth. Significant genetic and biochemical evidence supports the hypothesis that first eukaryotes originated from a chimeric organism, which arose after an unicellular archean endocytosed a symbiotic bacterium (most likely α-Proteobacterium), with the latter forming the original proto-mitochondrion. From this point on, the genomes of the two organisms slowly merged into a common system, giving mitochondria a central position in many basic cellular processes of modern eukaryotes (Martin et al., 2001; Baldauf, 2003; Saruhashi et al., 2008; Thiergart et al., 2012).

Mitochondria are central for many basic cellular processes of modern eukaryotes. Often likened to a "power plant," mitochondria are the site of oxidative phosphorylation, the biochemical process, which produces the vast majority of energy required for normal cellular function and survival in almost all eukaryotes (Gray, 2012; Papa et al., 2012). In most human cells, >90% of the energy required for survival is produced through mitochondrial respiration (Scheffler, 2008). However, production of energy is only one of many functions of this versatile organelle. Mitochondria play a central role in synthesis of basic structural lipids and sterols (Tatsuta et al., 2014), production of hemes and other critical cofactors (Heinemann et al., 2008; Fleming and Hamza, 2012). They also serve as a calcium store and a mediator of Ca^{2+} signaling (Rizzuto et al., 2012), act as a central switch in apoptotic and other cell-death pathways (Wang and Youle, 2009; Fatokun et al., 2014), and modulate many other critical cellular signaling cascades (Scheffler, 2008; Tait and Green, 2012).
1.1.2 Neurons require transport of mitochondria and a dynamic control of their structure and function

Normal activity of neuronal cells requires many highly specialized processes, such as synaptic vesicle recycling, or the activity of Na⁺/K⁺ pumps required for recovery ion gradients after action potential propagation. Many of these processes are very energy-intensive; in humans, for example, about 20% of the total energy expended is used by the brain, even though it comprises only a few percent of the total body mass (Tomasi et al., 2013). Mitochondrial respiration is required to cope with such high energy demand of neurons (Wong-Riley and Carroll, 1984; Bindokas et al., 1998; Laughlin, 2001; Guo et al., 2005a; Zinsmaier et al., 2009; Tomasi et al., 2013). Activity of mitochondria is also critical for many other basic functions of the neuron - synapse assembly, maturation, and even synaptic plasticity through neuron-specific Ca²⁺ modulation (reviewed in Zinsmaier et al., 2009; Sheng and Cai, 2012; also see further text).

In most somatic cells, high energy demands can be met simply by increasing the number of mitochondria, or their activity. However, neurons are highly polar and structurally complex cells, with long protrusions (axons and dendrites) which can exceed the size of the soma by orders of magnitude. For instance, some human motor neurons can have cell bodies measured in micrometers, while their axons can be over a meter long (Goldstein, 2003). Diffusion of ATP through such narrow processes is limited, and nowhere near sufficient to provide required energy to distant synapses (Hubley et al., 1996; Sun et al., 2013). Therefore, neuronal mitochondria must be actively transported to the distant compartments of axons and dendrites, and must be maintained and kept operational at a great distance from the soma. If mitochondrial transport is impaired, neuronal function and synaptic transmission will also fail as
soon as significant activity levels become required (Guo et al., 2005a; Sheng and Cai, 2012; Sheng, 2014). Proper distribution of mitochondria is also required for normal development and branching of various neurites (Courchet et al., 2013), and even transient presence of mitochondria can determine whether a particular nascent synapse will mature and stabilize (Sun et al., 2013).

How is mitochondrial transport accomplished? Most cells distribute mitochondria through multiple mitochondrial transport mechanisms, including myosin/actin-based transport, by attaching the organelles to polymerizing actin filaments, and by microtubule-based transport (Bridgman, 2004; Boldogh and Pon, 2006). Due to the long distances that need to be traversed, neurons rely exclusively on microtubule (MT)-based transport in their axons and dendrites, which is the fastest of the three (Morris and Hollenbeck, 1995; Ligon and Steward, 2000; Louie et al., 2008; Zinsmaier et al., 2009; also see further text). This exclusive requirement on MT-based transport generates a significant vulnerability to environmental and genetic stressors.

MTs consist of alpha- and beta-tubulin proteins, which polymerize into polar bundles beginning at the so-called minus-end and growing by polymerization towards the plus-end (Weisenberg, 1972). The orientation of MTs in axons or dendrites is important, since different motor proteins facilitate transport in opposing directions. Motors of the kinesin family transport cargoes towards the plus-end of MTs (with some exceptions; see Hirokawa and Takemura, 2005), while motors of the dynein family drive movements towards the minus-end (reviewed in Franker and Hoogenraad, 2013). Axons of most neuronal cells (fruit fly or mammalian) generally exhibit a (+)-end-out MT orientation, therefore requiring kinesin motors for antero- and dynein motors for retrograde transport (Franker and Hoogenraad, 2013). The situation is more complex in dendrites, which in some cases (such as mammalian hippocampal neurons) exhibit mixed MT polarity (Baas et al., 1988; Stepanova et al., 2003); but in other cases, dendrites can be
uniformly (-)-end-out oriented, as in *Drosophila* multidendritic sensory neurons (Rolls et al., 2007; Stone et al., 2008; Kollins et al., 2009). Regardless of whether the orientation is uniform or not, dynein-driven movements have been shown to be required for transport of various cargoes into dendrites (Satoh et al., 2008; Zheng et al., 2008; Kapitein et al., 2010). Therefore, direction of mitochondrial transport can be controlled by switching between opposing MT motors (Russo et al., 2009b; Zinsmaier et al., 2009). However, it still remains largely unclear how mitochondria "know" which direction they should choose. Two signaling pathways which arrest mitochondrial transport have been slowly uncovered. The first is based on the observations that mitochondria cluster in growth cones (Morris and Hollenbeck, 1993), which was later tied to nerve growth factor (NGF) signaling (Chada and Hollenbeck, 2003, 2004). When NGF is focally applied to neurons, mitochondria become attracted to the area, through a mechanism that increases their motility towards and decreases the motility away from the point of NGF application. This form of relatively long-range signaling appears to require filamentous actin, as well as TrkA kinase and phosphoinositide 3-kinase (PI3K) activity (Chada and Hollenbeck, 2003, 2004).

The second mechanism for control of mitochondrial motility is a much shorter range one, and alters mitochondrial distribution by simply stopping nearby mitochondria. In this case, local increases in Ca\(^{2+}\) concentration arrest dendritic mitochondrial transport through a mechanism which involves the mitochondrial GTPase Miro. The calcium-dependent immobilization allows mitochondria to concentrate in areas of the cell where synaptic activity (and therefore calcium influx) is relatively high (this pathway and the details of Miro function are discussed in section 1.3; also see MacAskill et al., 2009b; Wang and Schwarz, 2009). A third pathway for mitochondrial docking has been proposed, in which mitochondria are anchored to MTs by the protein syntaphilin (Kang et al., 2008). Recent studies, however, demonstrated that syntaphilin-
mediated MT docking acts through Miro calcium sensing mechanism, and therefore represents just a long-term modification of the same mechanism (Chen and Sheng, 2013; also see further text).

The complex architecture of neuronal cells produces several additional transport-related problems for mitochondrial biology, besides the need to transport mitochondria for local energy-production. Most mitochondrial genes are encoded within the genome of the neuron, and translated in the soma. Therefore, mitochondrial proteins or their respective mRNA must be must be somehow transported to mitochondria located in axons and dendrites to replace their damaged proteins. Similarly, replacement of lipids requires interactions with the ER, which contains many essential lipid-synthesizing enzymes. Finally, structural failure of mitochondria can release cytochrome c into the cytosol, potentially triggering an apoptotic cascade (Scheffler, 2008; Zinsmaier et al., 2009; Youle and van der Bliek, 2012).

In order to deal with these specific issues of mitochondrial biology in neurons, several additional mechanisms are closely interlaced with transport control. Individual mitochondria undergo continuous cycles during which they fuse with each other, sort the damaged components to one side, and fission apart - leaving one healthy organelle, and one with a higher accumulation of damaged proteins and lipids (see Youle and Narendra, 2011 for a short review; this process is described in detail in section 2.1). Once a mitochondrion accumulates enough damage that it can't maintain its membrane potential and normal function, it is degraded by a special form of autophagy. Mitophagy, as the process is called, requires de novo creation of a double-membrane envelope around the damaged organelle, which then fuses with a lysosome (mitophagy is described in detail in section 2.2; also see short review in Youle and Narendra, 2011). Little is know about autophagy/mitophagy in neuronal process except that its occurrence
may be limited, and that precursors of autophagosomes may be retrogradely transport to the soma.

Finally, mitochondrial fission involves an interaction with ER, where mitochondria "dock" at specialized sites. These docking sites regulate mitochondrial DNA replication and mitochondrial fission, exchange lipids, and perform many other functions (these interactions are described in section 1.2.3; also reviewed in Rowland and Voeltz, 2012).

All of these mechanisms (fission/fusion, mitophagy, ER docking) are interweaved with each other, and are mutually dependent on each other for proper function. When understood in this larger picture, mitochondrial transport can no longer be thought of as a simple unidirectional transfer of an energy-producing organelle from the soma to some distant outpost. Instead of just transferring mitochondria to their appointed place of service, mitochondrial transport also provides a critical logistical support system for mitochondrial function in axons and dendrites. For this purpose, mitochondria are transported in both directions, towards as well as away from the cell body. Some may be destined for relocation, others may carry fresh proteins from the soma, or carry damaged components back for degradation. It is difficult, if not impossible, to think of a mitochondrion in individual terms; instead, transport and fusion/fission cycles make all mitochondria of a cell a part of an overarching whole.

Unsurprisingly, problems with mitochondrial transport, function and morphology have been linked to many neurodegenerative diseases including (but not limited to) Alzheimer's disease, ALS, Charcot-Marie- Tooth disease, Friedrich's ataxia, Parkinson's disease, and others (De Vos et al., 2008; Koopman et al., 2012; Martin, 2012; Sheng and Cai, 2012). Recently, the main gene linked to heritable development of schizophrenia, DISC1 ("disrupted in schizophrenia 1"), was found to specifically bind and regulate mitochondrial transport machinery (Ogawa et al.,......
2014), indicating that control of mitochondrial transport may be important (or even causal) in at least some forms of this devastating disease.

1.2. Mechanism controlling mitochondrial structure and health

1.2.1 Mitochondrial fission and fusion

Mitochondrial cycles of fusion and fission are continuous and dynamic, even in resting cells (Lewis and Lewis, 1915; Johnson et al., 1981; Bereiter-Hahn and Voth, 1994). An increase in fusion creates longer organelles and reticular networks, while an increase in fission creates smaller, more grape-like mitochondria. Therefore, the exact shape and morphology in any given cell is determined by the balance between these two opposing mechanisms (Knott and Bossy-Wetzel, 2008; Chen and Chan, 2009). Interestingly, both fusion and fission are mediated by GTPases of the dynamin family, although their modes of action differ significantly (reviewed in van der Bliek et al., 2013).

The physical process of mitochondrial fission appears to be entirely driven by a single GTPase, dynamin-related protein 1 (commonly called Drp1, although the name appears as DnmL1, DLP1, or DVLP1 in older literature). Drp1 appears to be a universal feature of all eukaryotes, and is structurally and functionally conserved between yeast and metazoans (Bleazard et al., 1999; Labrousse et al., 1999; Smirnova et al., 2001; Praefcke and McMahon, 2004). Like dynamin, Drp1 assembles into large spiral multimers, albeit with a much larger diameter (~120 nm, compared to the dynamin's ~50 nm), which is required to accommodate both mitochondrial membranes (Ingerman et al., 2005; Chappie et al., 2010; Mears et al., 2011). Interestingly, unlike dynamin, Drp1 assembles as a quasi-double ("two-start") spiral, which appears to be required for generation of sufficient scissile force (Mears et al., 2011).
While Drp1 likely drives the “pinching” force behind the last stages of fission, it does not account for the entire process (Friedman et al., 2011). The average mitochondrion is 200-700 nm wide, far exceeding the ~120 nm diameter of the Drp1 helix. Therefore, some manner of additional constriction is required before fission can proceed. Currently, it appears that the ER may be involved in one such process (further discussed in section 2.3).

Drp1 also requires adaptor proteins, which show more variance between species. In yeast, a tetratricopeptide repeat protein Fis1 anchors itself in the outer mitochondrial membrane. There, it binds a cytosolic protein of the WD40 repeat family, Mdv1, which in turn recruits the yeast Drp1 homolog to the surface of the organelle (Mozdy et al., 2000; Tieu et al., 2002). Another WD40 protein, Caf4, is known to be partially redundant with Mdv1, and is also capable of recruiting Drp1 (Griffin et al., 2005). Mdv1 appears to be absent in higher eukaryotes. While Fis1 homologs exist in higher eukaryotes, they do not seem to be required for mitochondrial fission, but for mitophagy (Otera et al., 2010; also see further text). Recent work also shows that several different proteins (Fis1, Mff, Mid49, and Mid51) are all partially redundant in recruitment of Drp1 and promotion of fission (Niemann et al., 2005; Otera et al., 2010; Palmer et al., 2011). In addition, regulatory factors such as MIEF1 can bind and sequester Drp1, thereby promoting mitochondrial fusion (Zhao et al., 2011). It remains unclear how the inner mitochondrial membrane is divided, although it is possible that Drp1 scission divides both membranes at once.

Mitochondrial fusion presents a more complex picture, even though its mechanisms are fairly well understood (reviewed in van der Bliek et al., 2013). The first step is the fusion of the outer membrane, which is mediated by two monomers of the GTPase mitofusin. Situated on opposing membranes, two mitofusins dimerize and mediate fusion. It must be noted that the mechanism by which force is applied to fuse the membranes is not yet understood. In yeast,
fusion is performed by the GTPase Fzo1p (Hermann et al., 1998; Rapaport et al., 1998), which has homologs in mammals, *C. elegans* (FZO-1), and *Drosophila* (where the original mutation, fuzzy onions, was first discovered; see Hales and Fuller, 1997).

In *Drosophila*, Fzo1 is only expressed in the germline where it is required for fusion of germline mitochondria (male Fzo1 mutants are sterile). Mitochondrial fusion in other cells is mediated by a closely homologous and widely expressed protein Marf (Marf mutants are lethal; Dorn et al., 2011, also see further text). Mammals possess two proteins homologous to both Fzo and Marf, Mitofusins 1 and 2, which appear to be mostly redundant for mitochondrial fusion, but not for formation of mitochondria-ER contact sites (Santel and Fuller, 2001; Chen et al., 2003; also see section 2.3).

Once the outer mitochondrial membrane is fused, the inner membrane will almost always fuse as well (with a few reported exceptions, e.g. see Olichon et al., 2003). This process is mediated by another membrane-anchored GTPase, initially discovered in yeast through its effects on mitochondrial genome stability, and thus named mitochondrial genome maintenance 1 (Mgm1). Its human homolog was discovered independently as the mutant protein causing the inherited disease Dominant Optic Atrophy; based on the name of the disease, the protein is today called optic atrophy protein 1 (Opa1) in both flies and mammals (Jones and Fangman, 1992; Alexander et al., 2000; Delettre et al., 2000).

Mitochondrial fission and fusion serve two main roles: mitochondrial biogenesis and maintenance of a healthy mitochondrial population (Chan, 2012). Since mitochondria cannot be created *de novo*, new organelles are formed by replicating the mitochondrial genome and adding proteins and lipids to an existing one, which is then divided into two new parts (Scarpulla, 2008; Ventura-Clapier et al., 2008). Additionally, tightly linked fusion and fission events are important for mitochondrial quality control and the sorting of damaged proteins (Twig
et al., 2008b). Finally, fusion increases the polyploidy of the mitochondrial genome and allows mitochondria to deal with (relatively frequent) mutations. Accumulations of such mutations create a mixed population of mutant mitochondria, each with different deficiencies; through fusion, nucleoids, proteins and mRNAs can mix, allowing mutual complementation and restoration of functionality. This can allow cells to function even if 80 to 90% of their mitochondria contain compromised DNA (Yoneda et al., 1994; Nakada et al., 2001). In order to enhance complementation and functionality, fusion is directly enhanced when the cell encounters the need for more oxidative phosphorylation, or when under starvation conditions (Rossignol et al., 2004; Gomes et al., 2011; Rambold et al., 2011).

There is, however, only so much mitochondrial damage that can be complemented through fusion. The mitochondrial matrix and intermembrane space are among the biologically most hostile places within the cell. Up to 0.4% of all oxygen used in oxidative phosphorylation is converted into free radicals (Turrens and Boveris, 1980; Hansford et al., 1997), which damage surrounding DNA, proteins and lipids (Dai et al., 2014). While some of the protein damage can be dealt with individually through ubiquitin-proteasome degradation of damaged proteins (Tanaka et al., 2010), larger-scale degradation becomes necessary sooner or later. Thus, accumulating oxidative damage is dealt with in a manner that does not require wholesale destruction of organelles but repetitively sorts damaged components from many mitochondria into a few mitochondria designated for destruction.

Bacterial ancestors of modern mitochondria provide an effective strategy: they accumulate unfolded proteins and peroxidated lipids into a structure called an "aggresome," attach it to one side of the centrosome, and then divide asymmetrically. The daughter-cell that inherits the aggresome will be impaired, but the other daughter cells (and their progeny for
several generations, until another aggresome is needed) gain an advantage (Fuentealba et al., 2008; Lindner et al., 2008).

Mitochondria appear to utilize a similar strategy, although the mechanism by which it is achieved is not known. It has been shown that fission events occurring shortly after fusion generate two new daughter mitochondria, which significantly differ in their membrane potential; one displays a normal membrane potential (a measure of functionality), while the other shows a lower, impaired potential (Twig et al., 2008b). In fibroblasts, about one in five fission events results in a daughter-mitochondrion that is fully depolarized, and then eliminated through mitophagy (Twig et al., 2008b; mitophagy is discussed in detail in the next section). This strongly indicates that some kind of a protein sorting mechanism segregates the “good” and the “bad” proteins (it remains unclear what happens to the "ugly" proteins): prior to fission, one side of the mitochondrion appears to concentrate functional elements, while damaged and non-functional ones concentrate to the other side. By undergoing repetitive fusion/fission cycles and sorting damaged elements in each step, mitochondria could therefore continuously "rejuvenate" themselves, maintaining function while also continuously eliminating waste (Twig et al., 2008b; Twig et al., 2008a).

For both fission and fusion, coordination with mitochondrial transport (discussed in part 1.3 of this text) is of critical importance. After fission, two nascent smaller organelles have to be transported apart; for fusion, they need to be brought in close proximity with each other. Possible interactions of mitochondria with the ER (see part 1.2.3) also require coordination with the mitochondrial transport machinery.

Normal neuronal function imposes additional demands for fine-tuned control over the mitochondrial fission/fusion cycle. For instance, the ability to multiply mitochondria on demand and precisely transport them into dendrites, is critically important for the formation and
maturation of dendritic spines (Li et al., 2004). Control of mitochondrial shape and size is also required to properly distribute mitochondria into complex dendritic and axonal arbors, which is required for their normal outgrowth (Wakabayashi et al., 2009).

1.2.2 Mitochondrial degradation through mitophagy

The important role of mitochondria in apoptotic signaling produces a significant problem for their safe destruction. Structural failure of mitochondria can release many damaging factors into the cell, not the least the apoptosis-inducing cytochrome c and small mitochondria-derived activators of caspases (SMACs; Jiang and Wang, 2004). To avoid such devastating effects, disposal of damaged mitochondria is performed through mitophagy, a specialized pathway of autophagy.

Autophagy occurs through three main mechanisms (Mizushima and Komatsu, 2011). Microautophagy occurs when lysosomal membranes simply engulf portions of the cytoplasm, digesting any proteins or organelles present therein. Alternatively, in chaperone-mediated autophagy (CMA), the chaperone protein Hsc70 can recognize proteins which contain an exposed amino acid motif biochemically related to the pentapeptide KFERQ, and facilitate their translocation into the lysosome through interaction with the lysosomal receptor LAMP-2A (Orenstein and Cuervo, 2010; Mizushima and Komatsu, 2011).

The most common form of autophagy is macroautophagy, in which organelles, protein aggregates, or other subcellular entities are enveloped by a de novo generated double-membrane structure, the phagophore. The phagophore can develop in several different ways: in yeast, through coalescence of several smaller vesicles; in mammals, through a specialized extension of the ER (Axe et al., 2008; Mari et al., 2010). Alternatively (or additionally), membrane lipids may be supplied piecemeal from the ER, or even from the plasma membrane
itself (Hailey et al., 2010; Ravikumar et al., 2010). Once the phagophore fully encircles its target, it fuses, producing an autophagosome. The newly formed autophagosome then proceeds to either fuse with a multivesicular body (MVB), forming an amphisome; or with a lysosome, which leads to degradation of its contents. Each step of the process is regulated by numerous autophagy-related (Atg) proteins (reviewed in Nixon, 2013).

Mitophagy is a specialized form of macroautophagy specific to mitochondria, and does not require stressors such as starvation (although it can be induced by hypoxia). Instead, it is triggered by a mitochondrial membrane potential-dependent pathway that mediates the disposal of damaged mitochondria. This pathway removes damaged mitochondria, adjusts mitochondrial number in accordance with current metabolic needs, manages the steady-state turnover of mitochondrial membranes and components, and is required for certain stages in the development of some specialized mammalian cell types (Tal et al., 2007; Kundu et al., 2008). The enormous biological significance of maintaining mitochondrial quality through mitochondrial fusion & fission and mitophagy is illustrated by various neurodegenerative diseases, which can occur when these systems are impaired (Kim et al., 2007; Westermann, 2010).

A link between mitochondrial failure and neurodegeneration has been established many decades ago, when it was noticed that mitochondrial toxins (such as rotenone, paraquat or MPTP) induce acute parkinsonism (Langston et al., 1983; Schapira, 2008; Dagda et al., 2013). Additional studies demonstrated that both mitochondrial respiration and mitochondrial genome stability were impaired in the substantia nigra of Parkinson’s disease (PD) patients (Mann et al., 1992; Bender et al., 2006; Kraytsberg et al., 2006). Later, mutations in two genes responsible for the majority of recessive early-onset cases of familial PD were identified: the cytosolic E3 ubiquitin ligase parkin, and the mitochondrial serine/threonine kinase PINK1 (PTEN induced
PINK1 localizes to depolarized and dysfunctional mitochondria, and recruits parkin to them promoting mitochondrial mitophagy. Normal activity of parkin and PINK1 leads to degradation of damaged mitochondria and thereby suppresses the accumulation of such damage, which otherwise leads to loss of muscle, neurodegeneration, and male sterility in *Drosophila* (Greene et al., 2003; Park et al., 2006a; Deng et al., 2008; Poole et al., 2008). Knockout (KO) mice lacking Pink1, DJ-1 or Parkin genes show subtle behavioral and neurochemical abnormalities. For example, Pink1 KO mice exhibit decreased dopamine (DA) release in striatal slices and impairments in plasticity (Kitada et al., 2007) while Parkin KO mice exhibit increased striatal extracellular DA concentration and behavioral deficits (Goldberg et al., 2003). Details of how PINK1 and parkin facilitate mitophagy have been elucidated through a series of elegant studies.

PINK1 function is tightly linked to its import into mitochondria, which in turn depends on the mitochondrial membrane potential (health). If the membrane potential of a mitochondrion is normal, PINK1 is imported by the TOM complex of the outer mitochondrial membrane (OMM). Following import, it is passed on to the TIM complex of the inner membrane, where its charged signal sequence is rapidly cleaved off by the mitochondrial processing protease (MPP) in the mitochondrial matrix (Greene et al., 2003). The transmembrane domain of PINK1 in the IMM is then cleaved by presenilin-associated rhomboid-like protein (PARL) (Jin et al., 2010; Meissner et al., 2011) and the resulting 53 kDa fragment is retrotranslocated back into the cytosol. Once there, the 53 kDa fragment is recognized and degraded by the ubiquitin proteasome system, through the N-end rule pathway (Yamano and Youle, 2013). As a result, full-length PINK1 is
present on healthy mitochondria only at a very low level (Matsuda et al., 2010; Narendra et al., 2010b; Becker et al., 2012).

The functionality of the TIM import complex is dependent on the mitochondrial membrane potential (Endres et al., 1999; Wiedemann et al., 2004). Accumulation of damage eventually impairs the membrane potential, inhibiting TIM-mediated translocation of PINK1’s signal sequence into the matrix, which in turn prevents insertion of PINK1’s transmembrane domain into the IMM and subsequent PARL proteolysis. This leads to an accumulation of full-length and active PINK1 on the OMM (Lazarou et al., 2012; Okatsu et al., 2013), which then recruits parkin and promotes subsequent mitophagy.

In addition to depolarization-induced changes, some studies have shown that PINK1 may accumulate on the OMM in response to a buildup of misfolded matrix proteins (Thomas et al., 2014). In *Drosophila*, mutations in Lon protease (long undivided filaments upon UV irradiation), which degrades misfolded matrix proteins, led to a stabilization of full-length PINK1 on the OMM. The results suggest that Lon may also degrade PINK1 in the matrix, and that misfolded proteins may compete for this degradation pathway (Thomas et al., 2014). Similarly, in mammalian cells, expression of an unfolded protein (ΔOTC, deletion mutant of ornithine carbamoyltransferase) in the matrix increased PINK1 OMM levels, and downregulation of Lon increased them further. The authors, though, propose a different mechanism, in which unfolded proteins directly inhibit the TIM complex and thereby slow down PINK1 import and MPP/PARL proteolysis (Jin and Youle, 2013).

Once full-length PINK1 is enriched on the surface of mitochondria, it becomes active in its role as a serine/threonine-protein kinase, predominantly targeting ubiquitin. Specifically, PINK1 independently phosphorylates serine 65 (S65) of ubiquitin as well as S65 of parkin’s N-terminal ubiquitin-like domain (ULD) (Iguchi et al., 2013; Koyano et al., 2014). Both types of
phosphorylation are independent and partially sufficient for parkin recruitment to the mitochondrion, albeit optimal activation requires both (Kondapalli et al., 2012; Kane et al., 2014; Kazlauskaite et al., 2014a; Koyano et al., 2014). Interestingly, this process does not appear to be mitochondria-specific, since ectopic overexpression of PINK1 targeted to peroxisomes successfully induces pexophagy (Lazarou et al., 2012). Therefore, PINK1-induced autophagy is not dependent on mitochondria *per se*; instead, parkin-induced ubiquitination is a sufficient signal for autophagosomal formation.

Parkin’s affinity for phospho-S65-ubiquitin increases more than twentyfold after phosphorylation of parkin’s own S65 (Ordureau et al., 2014), which indicates that the system is built around a feed-forward loop. Namely, stabilization of full-length PINK1 on the OMM leads to S65 phosphorylation of ubiquitin chains on surrounding OMM proteins. This leads to recruitment of parkin to mitochondria, which is then further stabilized by PINK1 phosphorylation of parkin itself. Activated parkin then promotes the ubiquitylation of local substrates (Sarraf et al., 2013), producing new ubiquitin chains which are again phosphorylated by PINK1. These chains, in turn, recruit and stabilize more parkin on the OMM (Ordureau et al., 2014). The resulting cycle of extensive ubiquitination leads to degradation of mitochondrial surface proteins, stripping mitochondria of their ability to undergo transport (through degradation of Miro) and fusion (through degradation of mitofusin), but leaving their ability to undergo fission (reviewed in Pickrell and Youle, 2015).

Active parkin is capable of adding different types of ubiquitin chains to its many substrates on the OMM and in the cytosol (Chan et al., 2011; Narendra et al., 2012; Sarraf et al., 2013). Polyubiquitin chains linked through K11 or K48 promote extraction and proteosomal degradation of OMM proteins (including Miro and mitofusins) via the p97/VCP pathway, a process which appears to be required for mitophagy to proceed (Gegg et al., 2010; Poole et al.,
Parkin-generated K63-linked chains may be required for recruitment of the adaptor p62 autophagy receptor, linking the mitochondria with LC3 II, a critical autophagy receptor on the phagophore. However, deletion of p62 in mouse fibroblasts impaired only the clustering of ubiquitinated mitochondria (important for phagophore engulfement of multiple targets), but not their ultimate degradation. It therefore remains unclear whether such recruitment is truly required for mitophagy (Geisler et al., 2010; Narendra et al., 2010a; Okatsu et al., 2010).

Parkin also possesses a significant auto-ubiquitination activity, and the self-targeted ubiquitin chains appear to be mostly K6-linked. This noncanonical ubiquitin polymerization type has been shown to actually protect proteins from proteasomal degradation (Shang et al., 2005; Komander and Rape, 2012). Activity of deubiquitinase USP8 is required to remove these chains and allow normal turnover of parkin, which is in turn required for normal progression of mitophagy (Durcan et al., 2014). This function is quite distinct from other deubiquitinases known to interact with the PINK1/parkin pathway, all of which so far appear to slow down or arrest the mitophagy process (Bingol et al., 2014; Cornelissen et al., 2014).

While it seems probable that enrichment of (non-K6-linked) polyubiquitin chains marks mitochondria for degradation, as is the case for many other autophagy targets (Kirkin et al., 2009), the details of autophagosome recruitment remain unclear. Recent studies in mammalian cells (Yamano et al., 2014) and C. elegans (Shen et al., 2014) have shown that the fission protein Fis1 regulates autophagosome formation. Progression of the process appears to depend on two mitochondrial RabGAPs, TBC1D15 and TBC1D117, which bind to Fis1 directly (Yamano et al., 2014).
Beyond Fis1's dual role as an autophagosome adapter and a fission protein, there are other links between the mitochondrial fission/fusion cycle and mitophagy. As a general rule, fission precedes mitophagy in both yeast and mammals (Twig et al., 2008b; Westermann, 2010). In flies, overexpression of Drp1 or suppression of fusion (by heterozygous deletion of Opa1 or MARF) can suppress some phenotypes of PINK1 or parkin mutations (Deng et al., 2008; Poole et al., 2008). In yeast, Drp1 homolog Dnm1 is required for some mechanisms of mitophagy initiation (Frank et al., 2012; Abeliovich et al., 2013), and mitochondrial fission may facilitate or enhance engulfment by autophagosomes (Twig et al., 2008b; Tanaka et al., 2010; Buhlman et al., 2014). Mitophagy is therefore closely linked and coordinated with mitochondrial fission/fusion mechanisms.

Of particular interest is the PINK1/parkin pathway's ability to "bud off" small vesicles from mitochondria, carrying various types of oxidated and damaged molecules (Yang and Yang, 2013; McLelland et al., 2014). Passing such vesicles on to lysosomes allows for small-scale disposal and recycling of nonfunctional membrane-bound proteins, along with damaged or peroxidated lipids. This provides a potential pathway for the previously observed selective disposal of damaged ETC complex subunits (Vincow et al., 2013).

1.2.3 Functional significance of mitochondria-ER contact sites

Although interactions between mitochondria and ER have been studied for decades (Copeland and Dalton, 1959; Lewis and Tata, 1973), it was only in recent years that their importance for cellular function has been recognized. New biochemical techniques allowing isolation of the contact sites themselves (usually referred to as MAMs, for mitochondria-associated membranes) were critical for this development (Vance et al., 1997; Wieckowski et al., 2009).
At mito-ER contacts sites the two organelles come in very close proximity, with only 9-16 nm distance between membranes of mitochondria and smooth ER, or about 19-30 nm between mitochondria and rough ER (Rizzuto et al., 1998; Csordas et al., 2006; Goetz and Nabi, 2006). Once formed, these connections can be very stable, such that the two organelles can even be transported in tandem without disrupting the connection (Friedman et al., 2010; it should be noted that this "sliding" transport is different from normal mitochondrial transport described previously). While mito-ER contacts sites have been found in both yeast and metazoans (Ardail et al., 1990; Simbeni et al., 1991; Rizzuto et al., 1998; Simmen et al., 2005), their molecular organization and structure is drastically different (for review see Rowland and Voeltz, 2012).

In yeast, mitochondria tether to the ER through a protein complex named the ER-mitochondria encounter structure (ERMES). This complex consists of two OMM proteins, Mdm10 and Mdm34; the ER protein Mmm1; and the cytosolic protein Mdm12 (Kornmann et al., 2009; Stroud et al., 2011). These four proteins form a tight structure, which is required for normal cell health and survival (Kornmann et al., 2009).

None of the proteins of the yeast ERMES complex have metazoan homologs. Instead, one functional tether between mitochondria and the ER in mammals is the mitochondrial fusion protein mitofusin 2. While mitofusin 1 is entirely localized to mitochondria, a small fraction (~30%) of mitofusin 2 is present on ER and is capable of dimerizing with both mitofusin 1 and 2 on mitochondria (de Brito and Scorrano, 2008). Furthermore, mitofusin 2 is greatly enriched in the MAM fraction, as opposed to the overall ER membrane, indicating its association with the mito-ER bridging complex (de Brito and Scorrano, 2008). It is, however, worth noting that knockout of mitofusin 2 reduces the association between the two organelles by less than half (de Brito and Scorrano, 2008), which strongly suggests that other proteins are also involved.
Several interdependent functions have been discovered for mitochondria-ER contact sites. The MAM fraction provides clues to the first function, as it is enriched with enzymes involved in lipid synthesis (Vance, 1990). Synthesis of the most abundant structural lipids, such as phosphatidylethanolamine (PE) and phosphatidylcholine (PC) requires coordination between mitochondria and the ER, and the formation of contact sites. Phosphatidylserine (PS) is synthesized in the ER membrane (by PS synthase), and translocated through poorly understood mechanisms to the inner mitochondrial membrane (IMM), where it is converted into PE. Some of the PE is then transferred back to ER, where it is modified into PC. Since both mitochondrial membranes also contain PC, PC generated in the ER must be transported to the OMM and IMM (Zborowski et al., 1983; Ardail et al., 1993; Cui et al., 1993; Stone and Vance, 2000).

The second function of mitochondria-ER contact sites is regulation of ER-coupled mitochondrial fission, also known as ER-associated Mitochondrial Division (ERMD) (Friedman et al., 2011). While the scissile helix of Drp1 oligomers allows for a greater width than the narrow dynamin spiral, it is still far narrower than the normal width of the average mitochondrion. Hence, a constricting mechanism that precedes Drp1 recruitment is needed (Legesse-Miller et al., 2003; Ingerman et al., 2005). Indeed, observing the domains of interaction between ER and mitochondria, Friedman and colleagues showed that mitochondrial width becomes constricted in regions directly apposed to the ER (Friedman et al., 2011). Furthermore, Drp1 and its recruitment factor Mff then form punctate aggregations that overlap with sites of mitochondria-ER contact, although formation of contact sites and initial reduction in mitochondrial width is not dependent on the presence of Drp1 (Friedman et al., 2011). The constriction of mitochondria at sites of ER contact was further confirmed with high-resolution STORM microscopy (Shim et al., 2012).
Korobova and colleagues recently proposed an intriguing mechanism for pre-Drp1 changes in mitochondrial width. They identified ER-localized protein inverted formin 2 (INF2) as required for mitochondrial fission, acting upstream of Drp1. INF2 induced actin polymerization at the MAM sites, thereby constricting mitochondria and allowing Drp1 recruitment (Korobova et al., 2013). Interestingly, mutations in INF2, just like mutations in mitofusins, are known to cause some forms of Charcot-Marie-Tooth disease (Boyer et al., 2011).

ER-associated mitochondrial fission is also linked to mitochondrial DNA replication. Mitochondrial DNA (mtDNA) is present in multiple copies of circular nucleoids within the same organelle. Actively replicating nucleoids tend to associate with MAM sites (Hanekamp and Thorsness, 1996; Park et al., 2006b), and are otherwise limited in their ability to move through the organelle (Nunnari et al., 1997; Okamoto et al., 1998). In both mammalian and yeast cells, mtDNA nucleoids appear to accumulate at fission sites and mitochondrial tips, indicating that fission could be important for normal segregation of mtDNA (Garrido et al., 2003; Iborra et al., 2004). Recent studies have demonstrated that mtDNA nucleoids in yeast indeed replicate close to the ERMES foci, before being divided between the daughter-organelles through ER-associated division (Murley et al., 2013). Disruptions of core ERMES proteins have been shown to negatively affect nucleoid structure (Burgess et al., 1994; Youngman et al., 2004).

The third recently elucidated function of mitochondria-ER contact sites answers a long-standing question of mitochondrial calcium homeostasis. Namely, it has long been known that mitochondria accumulate large amounts of Ca\(^{2+}\) within their matrix, partially buffered by the formation of calcium phosphate complexes. Overall, mitochondrial Ca\(^{2+}\) levels exceed cytosolic Ca\(^{2+}\) by more than two orders of magnitude (reviewed in Pendin et al., 2014). While Ca\(^{2+}\) ions can easily pass through the OMM, passage through the IMM requires the action of the mitochondrial calcium uniporter, MCU, whose molecular identity was only recently discovered.
(Baughman et al., 2011; De Stefani et al., 2011). However, MCU's properties were well measured even before the protein was identified, and it was clear that it has a rather low affinity for Ca$^{2+}$ ($K_D$~20-50 μM), far too weak for efficient import from the cytosol (where Ca$^{2+}$ is generally held at ~100 nM even at maximal cytosolic concentrations; reviewed in Clapham, 2007). This presented a paradox: how can this low-affinity uniporter produce such repeatedly observed and massive changes in matrix Ca$^{2+}$ concentration? ER-mitochondria contacts offer a possible solution: the ER has an internal Ca$^{2+}$ concentration of ~1 mM, and ER Ca$^{2+}$ release can produce microdomains of high Ca$^{2+}$ in its close vicinity (Clapham, 2007). Indeed, the ER Ca$^{2+}$ channel inositol 1,4,5-triphosphate receptor (IP3R) has been shown to be a part of mitochondria-ER contact sites, and to physically associate with the OMM anion channel VDAC1 (Szabadkai et al., 2006). The close apposition formed by the IP3R-VDAC1 interaction is required for normal calcium transfer between both organelles (Szabadkai et al., 2006; Csordas et al., 2010). Hence, IP3R-driven Ca$^{2+}$ release may drive Ca$^{2+}$ uptake into the matrix through the MCU.

Once Ca$^{2+}$ is taken in by the mitochondria, it can be released again through the action of Na$^+$/Ca$^{2+}$ and H$^+$/Ca$^{2+}$ exchangers (Gunter and Sheu, 2009). These exchangers allow fine control of mitochondrial matrix Ca$^{2+}$ concentration (Gunter and Sheu, 2009; Palty and Sekler, 2012).

The Ca$^{2+}$ transfer affects two important aspects of mitochondrial function. First, since intramatrix Ca$^{2+}$ regulates the activity of the TCA cycle and enhances the activity of electron transport chain (ETC) complexes, changes in mitochondrial Ca$^{2+}$ load also alter the efficiency of ATP synthesis (Gellerich et al., 2010; Glancy and Balaban, 2012; Poburko and Demaurex, 2012). Secondly, mitochondrial Ca$^{2+}$ uptake regulates the calcium-dependent apoptotic pathway. Inhibition of IP3R Ca$^{2+}$ release through Akt kinase activity reduces mitochondrial Ca$^{2+}$
load and sensitivity to Ca\(^{2+}\)-induced apoptosis (Marchi et al., 2008; Szado et al., 2008); both are increased through activation of proapoptotic proteins Bax and FHIT (Chami et al., 2004; Rimessi et al., 2009). Recent studies have found that the tumor suppressor gene PML (named after promyelocytic leukemia) forms a complex with IP3R and regulates Ca\(^{2+}\) transfer and apoptosis. Deletion of PML causes IP3R phosphorylation, reduced Ca\(^{2+}\) efflux, and reduces apoptosis (Giorgi et al., 2010; Pinton et al., 2011). The mitochondrial apoptotic cascade also includes a feed-forward loop, as released cytochrome c binds to IP3Rs and increases Ca\(^{2+}\) efflux and mitochondrial Ca\(^{2+}\) import, further amplifying the apoptotic signal (Boehning et al., 2003).

Furthermore, recent work has implicated MAMs as the origin of at least some autophagosomal membranes. One study has identified OMM as the membrane source for autophagosome formation in conditions of starvation (Hailey et al., 2010), while another demonstrated that critical ATG proteins concentrate and specifically associate with MAM regions during initiation of autophagy (Hamasaki et al., 2013). The GTPase Rab32 associates with both ER and mitochondria, inducing the formation of membranous structures labeled with the autophagic marker LC3 (Hirota and Tanaka, 2009).

The MAMs have also been implicated in many other functions. For instance, mammalian target of rapamycin complex 2 (mTORC2) has been found to associate with MAM regions, possibly in order to coordinate mitochondrial function/lipid synthesis/autophagy with the overall metabolic state of the cell (Betz et al., 2013). MAMs also play an important role in control of immune reactions and inflammation (Zhou et al., 2011), important enough that some pathogens contain mechanisms that specifically target MAM-associated proteins (Horner et al., 2011). It is becoming clear that this evolutionarily ancient structure has been incorporated into many essential biochemical and physiological processes.
1.3. Mitochondrial GTPase Miro

1.3.1 General overview

The mitochondrial Rho GTPase Miro appears to be a universal eukaryotic gene with a few exceptions (Vlahou et al., 2011). For example, Miro is found in yeast (Wolff et al., 1999; Frederick et al., 2004), Drosophila (Guo et al., 2005a), mice (Shan et al., 2004), humans (Fransson et al., 2003; Fransson et al., 2006), and plants (Yamaoka and Leaver, 2008; Yamaoka and Hara-Nishimura, 2014). Of the named groups, yeast and Drosophila possess only one Miro gene (Gem1 and dMiro, respectively); vertebrates have two homologs, Miro1 and Miro2 (also known as RhoT1 and RhoT2), which appear to be partially redundant in function. The only eukaryotic organisms lacking Miro are the ones which also lack mitochondria, such as those belonging to genera Giardia or Trichomonas (Vlahou et al., 2011).

Structurally, Miro possesses a N-terminal GTPase domain (G1), two Ca\textsuperscript{2+}-binding EF-hand motifs, a C-terminal GTPase domain (G2), a variable domain, and a C-terminal transmembrane domain which tail-anchors the protein on the cytosolic side of the OMM (Figure 1; Wolff et al., 1999; Fransson et al., 2003; Klosowiak et al., 2013). Like other tail-anchored outer mitochondrial membrane proteins, Miro contains positively charged residues flanking the transmembrane sequence (Wattenberg and Lithgow, 2001; Borgese et al., 2003). While the functions of the GTPase and EF hand domains are discussed later, it is useful to reflect on their general nature.

The GTPase domains of Miro are related to small GTPases of the Rab/Ras family. This class of signaling GTPases generally hydrolyzes GTP slowly, and binds GDP very tightly. Accordingly, small GTPases typically require GTPase activating proteins (GAPs) to promote effective GTP hydrolysis, and guanine nucleotide exchange factors (GEFs) to facilitate GDP dissociation and GTP binding (for general reviews of the topic, see Bos et al., 2007; Cherfils and
Figure 1. Structure of Miro protein. G1, the N-terminal GTPase domain; EF1 and EF2, the calcium-binding EF-hand motifs; G2, the C-terminal GTPase domain; VD, the variable domain; and TM, the transmembrane domain.

Zeghouf, 2013). It is expected that Miro’s two GTPase domains have such cofactors as well, although none have been identified yet.

Miro was discovered three times independently. First, as a GTP- and Ca\textsuperscript{2+}-binding protein of an unknown nature (Wolff et al., 1999), then through a genomic search for previously uncharacterized Rho GTPases (Fransson et al., 2003), and finally by a genetic screen aimed at identifying novel synaptic proteins (Guo et al., 2005a). Miro was initially assigned to the Rho family of small GTPases (Fransson et al., 2003). However, Miro proteins lack the typical Rho-specific isoprenylation site, which anchors membrane-bound Rho GTPases to their target sites; instead, Miro is tail-anchored to the OMM via a full C-terminal transmembrane helix (Frederick et al., 2004; Fransson et al., 2006). More importantly, Miro’s GTPase domains lack the so-called "insert domain," an exposed alpha-helical domain that distinguishes Rho proteins from other members of the Ras superfamily and is important in downstream signaling (e.g. see Walker and Brown, 2002). As a result of these structural and functional differences, Miro proteins are today considered to constitute a subset of the Ras superfamily, independent of Rho (Boureux et al., 2007). It is worth noting that the similarity to Ras is mostly visible in the G1 domain, while the G2 is more divergent (Wennerberg and Der, 2004; Boureux et al., 2007).
EF-hands are well-known and well-characterized divalent ion binding helix-loop-helix motifs, first described over four decades ago (Kretsinger and Nockolds, 1973). While some versions of the motif are capable of binding either Ca\(^{2+}\) or Mg\(^{2+}\), many are highly specific to one ion. EF-hand variants are quite widespread in nature, with many hundreds of examples and dozens of protein families (reviewed in Nakayama and Kretsinger, 1994; Lewit-Bentley and Rety, 2000). Through dot-blot binding assays of \(^{45}\)Ca\(^{2+}\), EF-hand domains of Miro have been shown to be Ca\(^{2+}\)-binding variants (MacAskill et al., 2009b; also see Koshiba et al., 2011), and have proven to be important in mitochondrial transport control and Ca\(^{2+}\) homeostasis (see further text).

While two EF hands were identified in Miro proteins through sequence analysis (both canonical examples of the motif), a recently published partial structure of the protein (Klosowiak et al., 2013; the structure includes the EF-hands and the C-terminal GTPase domain) has found two additional cryptic EF-hand folds, bringing the number potentially to a total of four. Still, the solved structure shows only one EF-hand with bound Ca\(^{2+}\), and the functionality of the putative additional motifs remains unknown. Since most functional data have so far concentrated on the two canonical EF hands, this text will discuss only them; for clarity, the N-terminal EF-hand motif will be labeled EF1, and the C-terminal EF2.

Of particular note in the structural solution was the interaction between the EF2 motif and the G2 domain; the close intermeshing of the two strongly suggests that the switching state of the GTPase could influence the binding affinity of the EF-hand (Klosowiak et al., 2013). The opposing interaction, in which Ca\(^{2+}\) binding would influence GTPase activity, was excluded in previous biochemical studies (Koshiba et al., 2011). Furthermore, one of Miro's known PINK1 phosphorylation sites is buried within this interface (Klosowiak et al., 2013), and four ubiquitination sites are in close proximity. Disruption of the interaction between these domains
could, therefore, be important for PINK1/parkin-mediated Miro degradation during mitophagy (Klosowiak et al., 2013).

The variable domain of Miro was named for a region of the protein where alternative splicing produces three different protein isoforms. These isoforms differ only in the C-terminal sequence immediately preceding the transmembrane domain. Neither the protein sequence nor the length of this quasi-domain are conserved, and it has no recognizable functional or folding features (Babic and Zinsmaier, manuscript in preparation). However, the splicing site itself is highly conserved from flies to mammals, as is the presence of three distinct isoforms (this applies to Miro1 in mammals, and dMiro in *Drosophila*), which vary only in the length of the variable domain. The functional significance of this variation remains unclear, although it appears to have effects on mitochondrial health and structure (Babic and Zinsmaier, manuscript in preparation).

### 1.3.2 The function of Miro (Gem1) in yeast

When Gem1 mutants were first described in the literature, their main phenotypes were related to mitochondrial structure: while normal yeast mitochondria form a reticular network, loss of Gem1 caused fragmentation and collapse of the structure into individual globular, or grape-like, mitochondria. Loss of Gem1 also reduced respiratory function (Frederick et al., 2004). Both GTPase domains and both EF-hand motifs were required to fully rescue these phenotypes (Frederick et al., 2004).

Budding yeast replicates by budding off smaller daughter-cells (Herskowitz, 1988). Since mitochondria cannot be created *de novo*, the daughter cell needs to import some mitochondria from the mother, which requires active actin-based mitochondrial transport (Hermann and Shaw, 1998). Synthetic lethality was observed when Gem1 mutations were combined with
mutants of the known mitochondrial inheritance pathway proteins Mmr1 and Ypt11. Additional analyses suggested that Gem1, Mmr1, and Ypt11 may function in independent pathways to promote mitochondrial inheritance (Frederick et al., 2008).

While the mitochondrial inheritance function of Gem1 is important, the specific mechanism(s) underlying this role remained unclear. A series of elegant studies by Kornmann and colleagues shed light on pGem1’s molecular function, which is linked to the mitochondria-ER tethering complex (ERMES; see Figure 2). Initial identification of structural components of the ERMES complex identified Gem1 as a strongly interacting gene, and direct protein binding of Gem1 to the complex was later confirmed through tandem affinity purification and mass spectrometry (Kornmann et al., 2009; Kornmann et al., 2011). Gem1 is, however, not required for formation of the contact sites themselves (Kornmann et al., 2011).

Mutations that abrogate catalytic activity of Gem1's G1 domain also prevented the binding of Gem1 to the ERMES complex (Kornmann et al., 2011; Koshiba et al., 2011). A similar effect was observed for mutations which prevent Ca$^{2+}$ binding by Gem1's EF1 domain. Mutations in the EF2 and G2 domains had no significant effect. Therefore, only the N-terminal GTPase and the first of the two Ca$^{2+}$-binding domains of Gem1 are required for its proper localization to mitochondria-ER contact sites (Kornmann et al., 2011). However, both the G1 and G2 domain are required to rescue the synthetic lethality between Gem1 and Gep4, a phosphatidylglycerol-phosphate phosphatase that is essential for cardiolipin synthesis. Hence, while the G2 domain is not required for proper association of Gem1 with the ERMES complex, it certainly performs an important function in ERMES-related lipid synthesis and/or exchange regulation (Kornmann et al., 2011).

The ERMES complex itself is not a critical functional component of the machinery mediating phospholipid transfer between the ER and mitochondria. One study found lipid transfer
Figure 2. The Function of Miro in yeast. Yeast Miro’s (Gem1) G1 domain is required for association of Miro with the ERMES complex, which is in turn required for dissociation of mitochondria from the ER after ER-mediated fission. The G2 domain is involved in regulation of lipid synthesis.

and synthesis impairments in double mutants of both ER-shaping proteins and ERMES complex subunits (Mdm34); individual mutations in Mdm34 had no effect. This impairment could be rescued by expression of an artificial tethering protein between mitochondria and the ER, indicating that ERMES together with ER-shaping proteins like reticulons and atlastins provides a structural requirement, but not a lipid-transferring mechanism itself (Voss et al., 2012). Consistently, another study found no direct role for the ERMES complex and Gem1 in the transport of phosphatidylserine (PS) from the ER to mitochondria during the synthesis of phosphatidylethanolamine (PE), as PS to PE conversion is not affected in ERMES or Gem1 mutants (Nguyen et al., 2012). Hence, ERMES complex integrity is disposable for lipid exchange - as long as the contact sites are still made.
Figure 3. Miro is required for dissociation of mitochondria from the ER. (A), the initial phase of ER-mediated mitochondrial fission in yeast. Mitochondrion is associated with ER through ERMES complex. (B), ER wraps around and constricts the mitochondrion. The site of constriction is also associated with mitochondrial DNA replication. (C), Drp1 helix forms around the constricted mitochondrion, starting the actual fission of the organelle. (D), in wildtype animals, two organelles created through fission separate, each carrying its own contingent of replicated mtDNA. (E), in Miro mutants, the fission proceeds normally; however, the two resulting mitochondria both remain anchored to the ER, as the docking site fails to resolve.

What is the biological significance of the Gem1 association with ERMES if Gem1 is not required for ER-mitochondrial phospholipid transfer and/or synthesis itself? A recent study by Murley et al. analyzed mitochondria-ER interactions in regard to mitochondrial division, and found a strong link between ERMES complexes and sites of mitochondrial constriction and fission (Murley et al., 2013). In Gem1 mutant cells, ERMES complexes form and constriction of mitochondria occurs normally, but fails to resolve after mitochondrial fission is complete; fully separate, adjacent mitochondria remained in contact with the ER through ERMES-mediated connections. Loss of Gem1 function also caused consequences for mtDNA stability: nucleoids replicate close to the ERMES complex and the fission site, but without Gem1 activity, they remain in punctate aggregations close to the tethering complex. Therefore, it appears that
Gem1 may be required for proper mitochondrial dissociation from ERMES structures, and for proper resolution of the ER-associated mitochondrial fission process (Murley et al., 2013).

To summarize this using a simile useful for the remainder of this text: the yeast Miro homolog, Gem1, is required for normal undocking of mitochondria from their ER contact sites (Figure 3).

1.3.3 The function of Miro in metazoans

Despite the remarkable evolutionary conservation of the Miro GTPase family (Vlahou et al., 2011), divergent roles have been suggested for Miro in lower and higher eukaryotes. While yeast Miro is primarily required for mitochondrial homeostasis and the dissociation of mitochondria from ER contact sites, metazoan Miro is primarily required for mitochondrial transport, although it also has been implicated in mitochondrial homeostasis (reviewed in Lee and Lu, 2014).

The initial description of Miro in vertebrates showed that overexpression of a presumed constitutively active mutant of human Miro-1 (Miro-1/Val-13) induced an aggregation of the mitochondrial network and an increased apoptotic rate, indicative of a role in mitochondrial homeostasis (Fransson et al., 2003). However, soon afterwards, loss-of-function mutations in Drosophila Miro (dMiro) were discovered by a genetic screen for mutants which affect synaptic function. dMiro null mutant animals do not survive to adulthood, and die during 3rd instar-larval and pupal stages of development (Guo et al., 2005a). In dMiro null mutant neurons the distribution of mitochondria into dendrites and axons fails, with mitochondria accumulating within the soma (Guo et al., 2005a). However, the distribution of other synaptic cargoes is normal. Conversely, overexpression of dMiro caused an accumulation of mitochondria at the most distal
boutons of the neuromuscular junction (NMJ) (Guo et al., 2005a; Russo et al., 2009b). Together, this indicated that dMiro is required for axonal transport of mitochondria.

The complete loss of mitochondria at axon terminals of miro null motor neurons had a surprisingly modest effect on synaptic transmission (Guo et al., 2005a). Evoked neurotransmitter release was normal at low stimulation frequencies (≤1Hz), but could not be sustained at stimulation frequencies above 5 Hz for longer than 2 minutes, probably due to acute ATP depletion. Resting levels of presynaptic Ca$^{2+}$ at the NMJ were doubled, but activity-evoked Ca$^{2+}$ levels were not affected for short periods of high-frequency stimulation (Guo et al., 2005a).

The dMiro null mutant study also made a very peculiar observation: the lethality of dMiro null mutations could be rescued by neuron-specific expression of a Gal4-UAS driven cDNA of dMiro, but not by expression in other tissues. Therefore, dMiro function in fruit flies is essential only in neurons (Guo et al., 2005a). Notably, fly germ cells also critically require Miro since dmiro null mutant flies expressing normal dMiro in neurons were sterile, even though they had an almost normal adult lifespan (Guo and Zinsmaier, unpublished observations).

Detailed examination of mitochondrial movements in dMiro null mutants provided significant insights into dMiro’s role for distributing mitochondria (Russo et al., 2009b). In axons, mitochondria are transported in a semi-stochastic manner, often making short stops or changing the direction of movement. However, over a time period measured in minutes, every moving mitochondria seems to have a clear net-direction. Some move in a generally anterograde direction, away from cell body, by utilizing mostly (+)-end directed kinesin movements; others move in a generally retrograde direction, towards the cell body, by utilizing predominantly (-)-end directed dynein movements (Pilling et al., 2006). Loss of Miro has a peculiarly differential effect: for anterogradely-moving mitochondria, kinesin utilization is reduced, dynein utilization
unaffected, while mitochondria spend more time simply stopped; for retrogradely-moving mitochondria, dynein utilization was reduced, kinesin utilization unaffected, and the stops were again increased (Russo et al., 2009b). The implication of this result has been that dMiro is required for both kinesin and dynein motors. However, there is a caveat to this conclusion: the axons of dmiro null mutants contain very few mitochondria, which have a very unusual morphology (very small, very round); thus, it is possible that the observed dynein effects were secondary to a functional/structural problem with these mitochondria, and/or the failure of kinesin-dependent mitochondrial export into the axons.

The mitochondrial transport failure in fly Miro mutants was reminiscent of the phenotypes shown for mutants of the fly protein Milton, which also exhibited failure of anterograde mitochondrial transport in neurons (Stowers et al., 2002). Also similar to Miro, germ-cell specific deletion of Milton caused sterility due to failure of mitochondrial transfer to oocytes (Cox and Spradling, 2006). Milton protein colocalizes with mitochondria and directly binds the (+)-end directed microtubule motor subunit kinesin heavy chain (KHC) in Drosophila (Stowers et al., 2002). Further studies demonstrated that dMiro couples Milton and kinesin to mitochondria (Glater et al., 2006).

Mammals possess two homologs of Milton, which are now termed trafficking kinesin proteins 1 and 2 (TRAK1 and TRAK2) (next paragraph for details). Both proteins are about 56% homologous to Milton, and about 44% homologous to each other. Like Milton, both TRAK proteins are capable of binding kinesin (specifically KIF5) and Miro, and both are required for mitochondrial transport (Brickley et al., 2005; MacAskill et al., 2009a; Brickley et al., 2011; van Spronsen et al., 2013).

Unfortunately, independent discoveries have produced some confusing complications in the nomenclature of TRAK proteins. Both TRAK1 and TRAK2 were discovered as proteins that
Metazoan Miro serves as a mitochondrial anchor for motor proteins (kinesin and dynein) and their adaptors (TRAK1/2 in vertebrates, Milton in flies). Other proteins, such as OGT, are also known to be a part of the complex. Interact with the enzyme O-linked β-N-acetylglucosamine transferase (O-GlcNac transferase, or OGT) and therefore named OGT-interacting protein 106 and 98 (OIP106 and OIP98), respectively (Iyer et al., 2003). Independently, TRAK2 activity was found to be required for proper transport/localization of GABA<sub>A</sub> receptors, and named GABA<sub>A</sub>-interacting factor (GRIF-1) (Beck et al., 2002). Adding to the confusion, human TRAK1/2 were named hMilton1/2 in some studies (e.g. Pekkurnaz et al., 2014). To avoid exacerbating the issue, I will use the TRAK1/2 nomenclature exclusively.

An important side to the nomenclature confusion should be noted at this point: it shows that the TRAK-motor adaptor system is not exclusively used for mitochondria, but is also involved in transport of other cargoes, much in contrast to Miro. Indeed, besides transporting GABA<sub>A</sub> receptors, TRAK2 also binds Hrs and regulates endosomal trafficking; this activity is
required for ligand-dependent degradation of EGFR, a critical step in EGF signaling (Kirk et al., 2006).

Since vertebrate TRAKs bind both Miro and kinesin directly (as does Milton in Drosophila), the implied connection between Miro and dynein (Russo et al., 2009b) has long remained mysterious. A recent study demonstrated that TRAKs localize differentially in neuronal processes, with most TRAK1 found in axons, and most TRAK2 localized to dendrites (van Spronsen et al., 2013; Loss and Stephenson, 2015). Both TRAK proteins are able to bind p150Glued, a component of the dynein/dynactin complex, but only TRAK1 demonstrated a strong affinity for kinesin (van Spronsen et al., 2013). However, TRAK2 bound kinesin only when the motor protein was co-overexpressed (van Spronsen et al., 2013), which was also the case in many previous cell culture studies (Brickley et al., 2005; Brickley et al., 2011). Importantly, both TRAKs are equally capable of binding to Miro (van Spronsen et al., 2013).

An elegant series of experiments provided an explanation for the unusual interaction of TRAKs with kinesin (Figure 4; van Spronsen et al., 2013). Using rapamycin-mediated dimerization, it was demonstrated that TRAK2 predominantly assumes a "folded" conformation, with the N-terminal and C-terminal of the protein close together; this conformation facilitates dynein binding, but not the association with kinesin. TRAK1, however, was mostly in the "straight" conformation, in which kinesin binding was more efficient. Therefore, if folding of TRAK1 was induced, the kinesin affinity was lost, and the dynein affinity of the folded TRAK1 took over, changing the direction of transport (van Spronsen et al., 2013).

An obvious problem arises when this model of TRAK-motor binding is applied in Drosophila: while mammals possess two TRAK genes, flies have only one gene, milton. The mRNA products of the milton gene, however, can be differentially spliced to expresses several
protein isoforms. As the work in this thesis demonstrates (Chapter 3), two of these isoforms indeed appear to replicate the differential motor binding seen in the mammalian system.

Another complication arises from the surprising effects of the Miro1 gene knockout in mammals. Previously, Miro1 RNAi-mediated knockdown caused a severe reduction in the number of motile mitochondria in dendrites, but did not affect their speed (MacAskill et al., 2009b). However, conditional Miro1 gene knockout (KO) mice suggest that Miro1 is essential only for retrograde mitochondrial motility in axons, development and maintenance of specific cranial neurons, and for function of motor neurons. Loss of Miro1 was sufficient to cause neurodegeneration in mice without abrogating mitochondrial respiratory function (Nguyen et al., 2014).

Complete Miro1 KO was lethal at birth due to failure to breathe. However, conditional neuron-specific KO animals lived for about a month before succumbing to upper motor neuron pathologies. The percent of motile mitochondria was unaffected in Miro1 KO neurons axons, although the movement rate (number of mitochondria moving through a particular section of the axon) was reduced. This reduction was shown to be a result of reduction in dynein-based retrograde movements, while kinesin-based movements were unaffected (Nguyen et al., 2014). This was a startling reversal of many previous observations suggesting a Miro-kinesin link (Nguyen et al., 2014; the miro-kinesin complex is described in detail in further text).

It is possible that the apparently contradictory data stems from the activity of Miro2, since Miro2 overexpression can rescue Miro1 KO phenotype (Nguyen et al., 2014). Other differences between fly and mammalian observations, such as those in viability and mitochondrial motility, can also be explained by Miro2 activity. The dynein effect could be a result of differing affinities for TRAK protein complexes (see next paragraph). Certainly, more
work remains to be done to understand the role of both Miro homologs in mammalian mitochondrial transport.

1.3.4 Regulation of the metazoan Miro transport complex

The previously mentioned interaction between TRAK1/2 and OGT is an important regulator of mitochondrial transport. Mitochondria need to be able to adjust their movements so as to balance the need for energy production and the local availability of nutrients; without glucose or fat molecules, mitochondria don’t have anything to convert to ATP. It has been shown that OGT-dependent GlcNAcylation of TRAK1/2 increases in response to high levels of glucose, arresting mitochondrial transport. Thus, axonal mitochondria will accumulate in regions where higher levels of glucose are present (Pekkurnaz et al., 2014). Since OGT has also been shown to associate with Drosophila Milton, this mechanism is likely conserved (Glater et al., 2006).

More importantly for neuronal function, an interaction of Miro-Milton/TRAK complex with Ca$^{2+}$ signaling has been demonstrated in several model systems. Namely, mitochondrial transport can be reduced or stopped through local or global (micro-molar) increases in Ca$^{2+}$ levels (Yi et al., 2004; Chang et al., 2006). This adaptation allows mitochondria to concentrate at the synapses, providing the ATP needed to feed their high energy demand.

In both mammals and Drosophila, mutations in Miro’s EF-hand motifs completely prevent this Ca$^{2+}$-induced inhibition of transport (MacAskill et al., 2009b; Wang and Schwarz, 2009). In axons of cultured hippocampal neurons, EF-hand mutations cause increases in excitotoxicity at glutamatergic synapses, halving the number of neurons which can survive a NMDA challenge (Wang and Schwarz, 2009). Consistently, in dendrites of the same neurons, glutamatergic activation of the NMDA receptors was shown to be the cause of the Ca$^{2+}$ influx, which leads to
mitochondrial transport arrest (MacAskill et al., 2009b). Excitotoxic levels of Ca\(^{2+}\) influx apparently make mitochondria stop, which then contributes to neuronal loss.

The exact molecular mechanism by which Miro's EF-hands arrest mitochondrial transport appears to be different in dMiro and mammalian Miro (MacAskill et al., 2009b; Wang and Schwarz, 2009). In axons of cultured hippocampal neurons transfected with *Drosophila* KHC, Milton, and dMiro, increased Ca\(^{2+}\) levels caused kinesin disengagement from MTs, while the dMiro/Milton/kinesin complex remained intact (Wang and Schwarz, 2009). The authors proposed a model in which Ca\(^{2+}\)-bound EF-hands directly associate with the motor domain of KHC, which would disengage the mitochondrion from MTs and prevent further transport (Wang and Schwarz, 2009). In dendrites, the mammalian Miro/TRAK2 complex remains intact upon Ca\(^{2+}\) binding, but kinesin (KIF5) disengages from the mitochondria. The authors propose that KIF5 may associate directly with mammalian Miro, until this association is destabilized by Ca\(^{2+}\) binding to Miro's EF-hand motifs (MacAskill et al., 2009b). Thus, two fundamentally different modes of linking mitochondria to kinesin motors may exist in invertebrates and mammals.

Notably, Ca\(^{2+}\)-dependent arrest of mitochondrial transport in mammalian axons also requires the mitochondrial anchoring protein syntaphilin (Chen and Sheng, 2013). Syntaphilin is present in axons but almost absent in dendrites, and facilitates mitochondrial transport arrest through direct binding of kinesin upon Ca\(^{2+}\) influx, in a Miro-dependent manner (Chen and Sheng, 2013). Therefore, Ca\(^{2+}\) could induce dissociation of kinesin from mammalian Miro in both dendrites and axons - but since syntaphilin associates with the outer membrane of axonal mitochondria (Kang et al., 2008), axonal kinesin would remain bound to the mitochondrion while dendritic kinesin would dissociate fully.

The recent study on Miro1 knockout mice demonstrated that Miro1 deletion does not prevent Ca\(^{2+}\)-mediated mitochondrial transport arrest (Nguyen et al., 2014). However, as was
the case with other discrepancies between fly dMiro null phenotypes and those of mammalian Miro1 null, it is possible that Miro2's EF-hands take over for Miro1.

The role of metazoan Miro's GTPase domains has been less well explored than the role of their EF-hand motifs. Overexpression of Miro1 in COS7 cells, with a mutation (V13) which putatively constitutively activates its G1 domain, caused a collapse of the mitochondrial network and clustering of the organelles. Overexpression of Miro1 with an inactivated G1 domain (mutation N18) altered mitochondrial distribution and caused clustering (Fransson et al., 2003). While the V13 mutation of Miro2 exhibited the same clustering effect as seen for Miro1-V13, overexpression of analogous activating or inactivating mutations of the G2 domain of either Miro1 or 2 had no measurable effect on mitochondrial morphology (Fransson et al., 2006). Importantly, neither V13 nor N18 mutation affected binding of TRAK1 or TRAK2 to Miro (Fransson et al., 2006).

Two studies used the same G1 mutations to assess mitochondrial motility. Saotome and colleagues overexpressed Miro1 mutant proteins in primary cortical culture neurons, noting that the V13 mutation induced an increase in mitochondrial motility when Ca\(^{2+}\) levels were increased above physiological conditions; the G1-inactivating N18 mutation caused increased mitochondrial fragmentation (Saotome et al., 2008). Separately, MacAskill and colleagues overexpressed Miro1 GTPase mutants in developing hippocampal neurons, observing mitochondrial numbers in the peripheral processes. Overexpression of the Miro-N18 caused an increase of mitochondrial transport into the periphery of neurons, while Miro-V13 overexpression caused a decrease; both alterations resulted in a reduction of association between mitochondria and TRAK2 relative to cells overexpressing wildtype Miro (MacAskill et al., 2009a).
These results are difficult to reconcile and interpret, raising the possibility that overexpression may induce misleading phenotypes, or that the genetic nature of the mutations in Miro’s GTPase domain might be incorrect. Our recent studies confirmed this suspicion, and demonstrated that fly analogs of the Miro V13 mutation cause neomorphic gain of function effects that have little in common with the normal function of the protein (see Chapter 2).

1.3.5 Main questions addressed by this study

The work produced during this study was aimed at several open question of mitochondrial biology in neurons, focusing on the Miro-Milton transport complex. Chapter 2 focuses on the following questions:

- What are the main functions of the G1 and G2 domains of dMiro?
- Does dMiro control of dynein-driven transport of mitochondria?
- Is the function of Miro in metazoans truly distinct from its function in yeast?

Chapter 3 uses the insights from Chapter 2 and recent discoveries about the TRAK1/TRAK2 distinction (van Spronsen et al., 2013) to approach the following questions:

- Do Milton isoforms act in a manner analogous to TRAK1 and TRAK2 in mammals, differentially facilitating kinesin- and dynein-driven transport?
- Can Milton transport mitochondria independently of dMiro?
- Does dMiro's G1 domain control the association with different Milton isoforms?
CHAPTER 2: THE ROLE OF MIRO'S N-TERMINAL GTPASE DOMAIN IN MITOCHONDRIAL TRANSPORT

2.1 Summary

Previous studies have established that dMiro is critically required for axonal transport of mitochondria (Guo et al., 2005a; Russo et al., 2009b). Here, we explore whether this function requires the activity of dMiro's two GTPase domains, and further examine the role of dMiro in dynein-based mitochondrial transport in general. This chapter contains a short summary of the research; for the full study, please see Appendix A.

To determine the role of dMiro's two GTPase domains in mitochondrial transport, we generated dMiro cDNA transgenes containing established loss-of-function, as well as putative gain-of-function mutations. To inactivate dMiro's G1 domain, we introduced the amino acid substitution T25N; for the G2 domain, we used the substitution T460N. Homologous mutations have been shown to abolish GTPase activity in the yeast Miro homolog, Gem1 (Kornmann et al., 2011; Koshiba et al., 2011), and have been regarded as dominant-negative in mammalian studies (Fransson et al., 2003; Fransson et al., 2006; MacAskill et al., 2009a). To render the G1 and G2 domains constitutively active, we introduced substitutions A20V and K455V, respectively. These mutations were modeled after the constitutively active G12V mutation in Ras (Wittinghofer, 1998), and have been used as such in multiple studies (Fransson et al., 2003; Fransson et al., 2006; Saotome et al., 2008; MacAskill et al., 2009a). In addition, we also generated four double-mutant transgenes, containing all possible combinations of the activating and inactivating mutations (T25N-T460N, T25N-K455V, A20V-T460N, and A20V-K455V).

We used the Gal4 system to target the expression of these transgenes to motor or sensory neurons (MNs and SNs) of the Drosophila larvae (Brand and Perrimon, 1993). In motor
neurons, we took videos of mitochondrial movements, and analyzed their kinetics using previously established protocols (Louie et al., 2008; Russo et al., 2009b). We also quantified mitochondrial distribution in the neuromuscular junctions of mutant MNs, as well as in the dendritic arbors of mutant SNs.

Our results show that inactivation the G1 domain in dMiroT25N caused premature lethality and arrested development at a pupal stage, mimicking the dMiro null phenotype. Furthermore, dMiroT25N caused an accumulation of mitochondria in the soma of both larval MNs and SNs. Both the kinesin-dependent transport of mitochondria into axons, and the dynein-dependent transport into dendrites failed when G1 domain was inhibited. The few dMiroT25N-mutant mitochondria found in distal regions of the neurons were severely fragmented. These phenotypes were not affected by alterations in the G2 domain, as dMiroT25N was indistinguishable from both dMiroT25N-K455V and dMiroT25N-T460N.

We conclude that the G1 domain regulates mitochondrial transport irrespective of the employed motor, possibly by promoting transition from a stationary to a transport-competent state. Indeed, dMiro might serve the same "undocking from ER" function observed in yeast, with its effects on transport being a secondary metazoan adaptation.

Inactivation of the G2 domain through mutation dMiroT460N did not impair viability, mitochondrial distribution or mitochondrial morphology. Furthermore, the transport phenotypes of the G1 mutants dMiroA20V or dMiroT25N were unaffected by introduction of T460N mutation. However, dMiroT460N did somewhat reduce dynein motility during retrograde mitochondrial transport in axons. While we, therefore, cannot completely exclude the possibility of a minor modulatory role for the G2 domain in mitochondrial transport, it is likely that its main function lies elsewhere.
Importantly for interpretation of previous studies, the T25N mutation appears to be recessive, a result previously also seen in yeast (Koshiba et al., 2011). Therefore, studies which express Miro-T25N analogs in wildtype background may produce misleading results, as any changes may be due to simple overexpression effects of the other components of the protein (its role as a TRAK-kinesin anchor, for example).

The putative constitutively-active mutants A20V and K455V were both capable of facilitating mitochondrial transport into neuronal processes, much like normal dMiro. However, both mutants showed similar impairments in transport kinetics, as well as gross changes in mitochondrial morphology. Furthermore, dMiroA20V severely impaired viability, to less than 5%. The few animals that reached adulthood never lived longer than a day or two. All of these impairments were similar when mutants were expressed in wild-type or in dmiro null animals. Expression of double mutant A20V-K455V produced the same effects as the single mutants alone, as did the expression of the double constitutively active mutant A20V-T460N.

Since GTPase signaling is not supposed to operate continuously, it is possible that these effects are a result of negative feedback responses attempting to counteract the chronic activation of the domain. Still, the fact that addition of wild-type protein does not modulate the effects of the mutant protein argues somewhat against such explanations, as does the lack of additive effects upon expression of the double constitutively active mutant A20V-K455V. A likely explanation is that these mutations actually cause a neomorphic phenotype, through an acquisition of a toxic protein feature or an unnatural interaction with other factors.

In summary, our study shows that dMiro’s N-terminal GTPase domain is critically required for viability, for control of mitochondrial morphology, and for both kinesin- and dynein-driven mitochondrial motility.
CHAPTER 3: MILTON ISOFORMS CONTROL BOTH KINESIN-AND DYNEIN-DRIVEN MITOCHONDRIAL TRANSPORT IN DROSOPHILA NEURONS

3.1 Abstract

Microtubule-based transport of mitochondria into dendrites and axons is critical for normal synaptic function. Kinesin motors drive microtubule plus-end-directed transport of mitochondria, while minus-end-directed transport is driven by dynein. However, the mechanisms controlling the selective use of these motors remain poorly understood. In mammals, kinesin and dynein are linked to mitochondrial Miro through the adaptor proteins TRAK1 and 2, respectively. We show that the only Drosophila homolog of TRAK1/2, Milton, facilitates not only kinesin- but also dynein-driven transport in axons. Milton expresses four different protein isoforms (A-D) generated by alternative mRNA splicing. Acute overexpression of Milton-A accumulates mitochondria at axon terminals within 24 hours, while acute overexpression of Milton-B depletes axons of mitochondria and accumulates them in cell bodies and dendrites. Furthermore, we show that the activity of both Milton isoforms is strictly dependent on the presence of Miro, and that it requires activity of Miro's N-terminal GTPase domain. Beyond extending mammalian findings to Drosophila, our findings further support the role of Miro in facilitating a transition of mitochondria from a stationary to a motile state. Differential binding of Milton-motor complexes presents a potential candidate mechanism for determining the direction of mitochondrial transport in neurons.
3.2 Introduction

Neurons are highly differentiated and polarized cells, with axons and dendrites that can extend to distances orders of magnitude greater than the diameter of their cell bodies. Many of the most energy-demanding processes required for neuronal function, such as synaptic transmission, occur at significant distances from the soma. To address these energy needs, mitochondria must be transported dynamically and precisely to these distant compartments. Complicating the problem, disposal of damaged mitochondria and maintenance of their health also requires transport back towards the cell body. Impairments of this transport system are linked to a number of neurodegenerative diseases (for review see Mattson et al., 2008; Zinsmaier et al., 2009; Saxton and Hollenbeck, 2012; Sheng and Cai, 2012).

In all studied metazoan species, mitochondrial transport in axons and dendrites relies on microtubules (MTs) and MT motors of the kinesin and dynein families (reviewed in Franker and Hoogenraad, 2013). Kinesins generally drive transport towards the plus end of MTs, while dyneins drive transport towards the minus end (Boldogh and Pon, 2006; Pilling et al., 2006; Saxton and Hollenbeck, 2012). Regulation of these opposing motor activities is critical for the ultimate distribution of mitochondria in neurons; and their differential control is required for sorting of cargoes between different compartments of the neuron (Kapitein et al., 2010; Saxton and Hollenbeck, 2012).

In metazoans, a central player in mitochondrial transport is the atypical mitochondrial GTPase Miro. Tail-anchored on the cytosolic side of the outer mitochondrial membrane, Miro proteins usually possess two GTPase domains and two Ca\(^{2+}\)-binding EF-hand domains (Vlahou et al., 2011; reviewed in Lee and Lu, 2014). Miro is an important structural part of the mitochondrial transport complex, implicated by multiple studies as the main anchor for association of kinesin motors with mitochondria (Glater et al., 2006; MacAskill et al., 2009a). Its
EF-hand domains play a crucial role in calcium- and neuronal activity-dependent arrest of mitochondrial transport, by regulating kinesin association with MTs and/or with mitochondrial transport complexes (MacAskill et al., 2009b; Wang and Schwarz, 2009). Furthermore, our recent study shows that Miro’s N-terminal GTPase domain acts as a general on/off switch for mitochondrial motility, and that abolishment of its activity largely prevents both kinesin- and dynein-driven mitochondrial transport (Chapter 2).

In vertebrates, the linkage between mitochondrial Miro and kinesin or dynein motors is mediated by the adaptor proteins TRAK1 and TRAK2, respectively (Fransson et al., 2003; Brickley et al., 2005; Fransson et al., 2006; Koutsopoulos et al., 2010; van Spronsen et al., 2013). Both TRAKs bind to Miro on mitochondria but exhibit different motor-binding affinities, with TRAK1 predominantly facilitating kinesin-mitochondria association in axons, while TRAK2 predominantly facilitates dynein-mitochondria association in dendrites (van Spronsen et al., 2013). *Drosophila* possess only one TRAK1/2-homologous gene, *milton*. Milton is critically required for anterograde mitochondrial transport, and can bind Miro and kinesin heavy chain (KHC), linking kinesin with mitochondria (Stowers et al., 2002; Glater et al., 2006). The mechanism by which dynein interacts with this Miro-Milton complex is unknown.

We now show that two isoforms of Milton protein that are derived by alternate mRNA splicing differentially affect mitochondrial distributions in axons and dendrites. Overexpression of Milton-A in motor neurons caused rapid mitochondrial redistribution towards the neuromuscular junction, which can only be achieved by promoting kinesin-based transport. Conversely, overexpression of Milton-B caused a rapid redistribution from the NMJ towards the soma, consistent with an increase of dynein-based transport. We further show that both effects of Milton A and B OE are dependent on presence of Miro, and on the activity of Miro’s N-terminal GTPase domain.
Our results show that the mammalian TRAK1/2 differential motor binding mechanism is conserved in *Drosophila*, and further support a model in which Miro’s N-terminal GTPase domain regulates a transition of mitochondria from a stationary to a motile state.
3.3 Methods

Fly Stocks

For chronic expression experiments, flies were raised on standard cornmeal medium with dry yeast at 22°C. For acute expression experiments, larvae were raised on grape juice plates for 96 hours, and then transferred to grape juice plates containing 10 µg/mL mifepristone for ~24 hours. The strain expressing green fluorescent protein (GFP) tagged by an N-terminal mitochondrial localization signal (\(w^{1118}, P[w^+]; UAS-mitoGFP\)) was obtained from W. Saxton (University of California Santa Cruz, Santa Cruz, CA). The mutants \(dmiro^{SD32}\) (deletion of 29 bp at Y89) and \(dmiro^{B682}\) (point mutation W105/stop) prematurely truncate dMiro protein within the N-terminal GTPase domain, and are considered null mutations (Guo et al., 2005b; Russo et al., 2009a). For chronic expression of UAS transgenes in motor neurons, we used the OK6 driver (Aberle et al., 2002). Acute expression was accomplished using GeneSwitch drivers 176-Gal4 for motor neurons, and 734-Gal4 for sensory neurons (Roman et al., 2001; Nicholson et al., 2008).

For all experiments, strains containing a homozygous UAS-Milton transgene in an otherwise wild type or heterozygous \(dmiro\) null mutant genetic background (\(w^{1118}, P[w^+], UAS-Milton-X\) or \(w^{1118}, P[w^+], UAS-Milton-X; dmiro^{B682}/TM6TbSb\)) were crossed to strains containing a homozygous Gal4-driver and a UAS-mitoGFP transgene in a wild type or heterozygous \(dmiro\) null background (\(w^{1118}, P[w^+], Gal4-driver\); \(P[w^+], UAS-mitoGFP\) or \(w^{1118}, P[w^+], UAS-mitoGFP\), \(dmrio^{SD32}/TM6TbSb\)) to generate progeny that express a single copy of the UAS-transgene driven by the Gal4 driver in the respective genetic background (\(w^{1118}, P[w^+], Gal4-driver\)/\(P[w^+, UAS-Milton-X]; P[w^+, UAS-mitoGFP]/+\) or \(w^{1118}, P[w^+, Gal4-driver]/P[w^+, UAS-Milton-X]; dmiro^{SD32}, P[w^+, UAS-mitoGFP]/dmrio^{B682}\)).
Generation of Milton mutant transgenes

We PCR amplified the DNA sequence of Milton-A from cDNA LD08974 (Drosophila Genomics Resource Center, DGRC) using the primers Milton-A forward and Milton-both reverse (see below). The sequence of Milton-B was PCR amplified from cDNA LD33316 (DGRC) with primers Milton-B forward and Milton-both reverse.

Milton-A forward 5’AATTCCATTTAATGCTATCCGCAACTTTGGGGGCCAATGGCG3’
Milton-B forward 5’ GAATTCGACAAAATGACGCACGTAAACAATGGGGAAGTAATGGAAAAGGAG3’
Milton-both-reverse 5’AGGTACCTACAGCCAACCGCCCTTGCGGCC3’

The PCR products were directionally cloned into NotI and KpnI cleaved pUAST vector (Brand and Perrimon, 1993), and their integrity confirmed by DNA sequencing. pUAST plasmids were injected into w1118 embryos, generating transgenic animals (Rainbow Transgenic, Camarillo, CA). At least two independent strains were obtained for each transgene. After outcrossing all other chromosomes, strains containing homozygous UAS-transgenes on the 2nd chromosome were generated containing a wild type-like genetic background (w1118) or a dmiro null mutant background (dmiroB682).

Immunostainings

Climbing third-instar Drosophila larvae were dissected in HL-3 solution and fixed in 4% PBS-buffered paraformaldehyde (pH 7.3). After three 10 minute washes in PBS-T (PBS with 0.2% Triton X-100, pH 7.3), the preparation was incubated with primary antibody overnight at 4°C, washed 3x in PBST for 10 minutes, and incubated with secondary antibody for 2 hours at room temperature. After final washing, the preparations were post-fixed for 10 minutes in 4% PBS-buffered paraformaldehyde (pH7.3). Confocal images were acquired within 48 hours after post-fixation. The following antibodies and dilutions were used: goat anti-HRP-Cy3 at 1:300 (Jackson
ImmunoResearch, #123-165-021); rabbit anti-GFP-AF488 at 1:1000 (Molecular Probes, #A21311 RRID:AB_221477); mouse anti-ATPSynthase (ATP5A, Abcam, #ab14748, RRID:AB_301447) at 1:1000; anti-mouse-Cy3 at 1:1000 (Jackson ImmunoResearch, #715-165-150); and goat anti-HRP-AF647 at 1:500 (Jackson ImmunoResearch, #123-605-021, RRID:AB_2338967).

Quantification
To determine the mitochondrial density at NMJs of larval MNs and dendrites of SNs, respective confocal images were obtained from dissected 3rd instar larvae that were immunostained for mitoGFP-labeled mitochondria and neuronal membranes (anti-HRP). For analysis of NMJs, images were obtained from muscles 6/7 in segment 3-4. The area of a synaptic bouton marked by HRP and the respective area of the bouton occupied by mitochondria marked by mitoGFP was measured using ImageJ software to determine the fraction of the synaptic area occupied by mitochondria. For analysis of occupancy, number of mitochondria-occupied and unoccupied boutons was counted. For analysis of dendrites, images of vpda sensory neurons were obtained from segment A2. The length of primary and secondary dendrites and the number of mitochondria in the respective dendrites was measured using ImageJ software. The amount of dendritic mitochondria was normalized to dendritic length. For mitochondrial size measurements, areas of individual mitochondria were measured using ImageJ. Statistics and data analysis were performed with Microsoft Excel and GraphPad Prism.
3.4 Results

In mammals, the adaptor protein TRAK1 binds to both kinesin and Miro, linking kinesin to mitochondria, while TRAK2 facilitates dynein linkage (van Spronsen et al., 2013). Since the *Drosophila* genome contains only one TRAK1/2-homologous gene, *milton* (Stowers et al., 2002; Glater et al., 2006), we tested whether different isoforms of Milton may exhibit opposing motor-binding affinities similar to TRAK1 and 2. The *Drosophila milton* gene expresses at least six alternatively spliced and distinct mRNA transcripts, which are translated into four distinct protein isoforms (Fig. 5A). While the majority of the protein sequence is identical for all four isoforms, three isoforms (A, B and C) have different N-terminal sequences (~140 aa, Fig. 5B), which are translated from completely distinct exons.

We excluded Milton-C and –D from further consideration. Milton-C is a genus-specific transcript, since its N-terminal sequence is unique to Drosophilidae and not found outside this group. Milton-D is also an unlikely candidate, since the transcript has been found only in testicular libraries (Stowers et al., 2002; Glater et al., 2006). On these assumptions, we speculated that Milton-A and Milton-B are the best candidates for mediating opposing functions in mitochondrial transport, and constructed Gal4/UAS-controlled transgenic lines capable of expressing these two isoforms.

Chronic overexpression of Milton-A and -B isoforms had a strong effect on viability. Pan-neuronal overexpression of Milton-A using an elav-Gal4 driver was lethal during the late embryonic to early first-instar stage of development. OK6-Gal4 driven overexpression of Milton-A in motor neurons and a few interneurons of the ventral nerve cord was lethal during pupal stages. In contrast, both pan-neuronal and motor neuron overexpression of Milton-B was lethal during the pupal stage.
Chronic overexpression of Milton-A in motor neurons causes mitochondrial destruction

We used the OK6-Gal4 driver to chronically overexpress each isoform of Milton in motor neurons of wandering *Drosophila* third-instar larvae. To label mitochondria, we co-expressed mitochondrially-targeted GFP (mitoGFP). The axons of *Drosophila* neurons are known to contain uniformly organized plus-end-out MTs (Rolls, 2011), such that anterograde
mitochondrial transport in the axon requires kinesin (KHC) motor activity (Fig. 5C; Pilling et al., 2006). Similarly, any retrograde transport from the synapse towards the cell body requires dynein motor activity (Pilling et al., 2006).

Expression of mitoGFP in otherwise wild type flies strongly labels mitochondria as discrete punctae or a reticular network, such that mitoGFP fluorescence is not detectable in the cytosol (Fig. 6, Pilling et al., 2006). Surprisingly, in motor neurons overexpressing Milton-A mitoGFP labeling was markedly different. In particular, most mitoGFP fluorescence was cytosolic instead of being limited to mitochondria (Fig. 6, arrowheads).

In addition, Milton-A OE severely reduced the number of mitochondria present in the soma, dendrites and axons (Fig. 6). The remaining mitochondria formed large irregularly shaped aggregations, very distinct from grape- or worm-like shapes of wild type mitochondria (Fig. 6, arrows). At the NMJ, the few remaining mitochondria were small and globular, and a significant proportion of boutons lacked any mitochondria (Figs. 6, 7G). Thus, chronic overexpression of Milton-A uniformly reduces the number of mitochondria throughout the cell, and induces mitochondrial aggregation and degradation through an unknown mechanism.

**Acute overexpression of Milton-A induces a redistribution of mitochondria from the soma to the most distal tips of axons**

The cell-wide loss of mitochondria caused by chronic overexpression of Milton-A made it difficult to examine its effects on mitochondrial transport. To get around this problem, we acutely overexpressed Milton-A by using the GeneSwitch motor neuron driver 176-Gal4, which is activated by feeding application of mifepristone (10 µg/mL; Nicholson et al., 2008). Mifepristone activates the GeneSwitch Gal4 driver, inducing the expression of the transgenic UAS-Milton construct (Nicholson et al., 2008). After 96 hours of development in the absence of application
of the drug, we induced coexpression of Milton-A/B and mitoGFP by transferring the larvae to mifepristone-containing plates for 24 hours. This period was chosen as approaching the minimal amount of time required for sufficient expression of mitoGFP marker.

Acute overexpression of Milton-A for 24h exhibited signs of early mitochondrial destruction (cytosolic mitoGFP, Fig. 7A arrowheads), but also severely reduced the number of mitochondria in the motor neuron somata (Fig. 7A,C) and increased the amount of mitochondria at the most distal boutons of NMJs in comparison to control (Fig. 7A,E-F). Milton-A OE caused an increase in the size of terminal boutons (data not shown) and doubled the fraction of the terminal boutons occupied by mitochondria (54% vs. 27% in control, p<0.001, Fig. 7F). Acute
Figure 7. Effects of acute Milton-A and Milton-B overexpression on mitochondrial distribution in motor neurons. (A-H), mitoGFP was transgenically coexpressed with Milton-A or Milton-B, using the chronic OK6-Gal4 driver (G), or the 24 hour acute 176-Gal4 driver (A-F,H). Mitochondria were visualized by confocal imaging of immunostained larval filet preparations, using anti-GFP, anti-HRP and anti-ATPSA antibodies. Asterisks indicate significant differences among indicated genotypes (Kruskal-Wallis with Dunn's post-test for datasets which failed to pass D'Agostino&Pearson omnibus normality test; one-way ANOVA with Brown-Forsythe for others). (A), Mitochondria (green) in VNC (top), soma and neuropil (middle) or NMJ (bottom) of wt MNs, and MNs acutely overexpressing (176-Gal4 GeneSwitch, expression induced for 24 hours at age of 96 hours) Milton-A or Milton-B. Arrowheads indicate cytosolic mitoGFP in somata of Milton-A overexpressing neurons, suggesting a severe loss of mitochondria. Arrows indicate mitoGFP aggregations. Scale bar: 45 μm for VNC, 25 μm for soma and NMJ. (B), mitochondria (green and red) at the NMJs of wildtype larvae, and at NMJs of animals chronically (OK6) overexpressing Milton-B. (C-D), intensity of mitoGFP signal in somata (C) or neuropil (D) of MNs (p<0.05, N>5). (E-F), mitochondrial area of (E) all NMJ boutons or (F) terminal boutons only (p<0.001, N>5). (G), percent of NMJ boutons occupied by mitochondria (p<0.05, N>5). (H), Intensity of ATPSAl staining within the NMJ boutons (p<0.01, N=4).
Milton-A OE did not have a significant effect on the amount of mitochondria in en passant boutons (36% vs. 31% in control, p>0.99, Fig. 7E). The Milton-A OE induced redistribution of mitochondria from the soma to the most distal tips of axons suggests that Milton-A may predominantly mediate anterograde kinesin-based axonal transport.

**Overexpression of Milton-B in motor neurons induces a redistribution of mitochondria from the NMJ to the soma**

In contrast to Milton-A, chronic overexpression of Milton-B did not cause a pronounced loss of mitochondria. Instead, Milton-B expression severely altered the mitochondrial distribution in motor neurons such that the mitochondrial occupancy of distal axons (not shown) and NMJs was reduced to 17.6% of control (p<0.0001, Figs. 6, 7G) while mitochondria accumulated in the soma and neuropil (Fig. 6, arrows). Milton-B OE had no apparent detrimental effects on the integrity of mitochondria since cytosolic GFP was not detectable. This OE effect is consistent with the idea that Milton-B may predominantly facilitate dynein-driven retrograde axonal transport. Alternatively, the induced axon-to-soma redistribution of mitochondria could indicate a defect in facilitating and/or initiating anterograde axonal transport. To distinguish between these possibilities, we overexpressed Milton-B acutely.

Acute overexpression of Milton-B for 24 hours also caused an accumulation of mitochondria in the motor neuron soma (amount of mitoGFP signal doubled in comparison to control, p<0.001, Fig. 7A, C-D), and a depletion at the NMJ (mitochondrial area of boutons reduced to nearly zero, p<0.001, Fig. 7A, E-F). In addition, Milton-B OE caused large axonal accumulations of mitochondria in the neuropil of the VNC, at a position that corresponds to the areas where the primary neurite branches into a single axon and multiple dendrites (Figs. 5C, 7A arrows, 7D).
Figure 8. Effects of Milton-B overexpression on mitochondrial distribution in sensory neurons. (A-G), mitoGFP was transgenically coexpressed with Milton-B for 24 hours, using the acute 734-Gal4 driver. Mitochondria were visualized by confocal imaging of immunostained larval filet preparations, using anti-GFP and anti-HRP antibodies. Asterisks indicate significant differences among indicated genotypes (Mann-Whitney, or unpaired Student's t-test). (A-B), mitochondria (green) in dendrites of vpda multidendritic sensory neurons. Arrowheads mark the primary dendrite, arrows note some of the secondary dendrites. Squares indicate detail areas shown to the right of each neuron. Note the increased size of the mitoGFP signal in Milton-B OE dendrites (B) and the concomitant expansion of the dendrite. (C), total number of dendritic mitochondria (p>0.05, n=6). (D), number of mitochondria in primary dendrites (p<0.001, n=6). (E), number of mitochondria in secondary dendrites (p>0.99, n=6). (F), size of dendritic mitoGFP punctae (p<0.001, n=4). (G), size distribution of dendritic mitoGFP punctae. Scale bars: 10 µm (full SN images), 20 µm (detail).
To verify that mitochondria at Milton-B overexpressing NMJs were indeed absent instead of being incompletely stained by the co-expressed mitoGFP tag, we double stained the NMJ using antibodies against the mitochondrial subunit ATP5A of the ATP Synthase (Capaldi et al., 2004) and HRP which stains the neuronal membrane (Fig. 7B). Even though the strong staining of mitochondria in the muscle contaminates measurements of ATPSynthase fluorescence contained in synaptic boutons, Milton-B overexpression reduced ATPSynthase fluorescence to 55% of control (p<0.001, N=4, Fig. 7H). There was also a significant difference between boutons of NMJs where expression of Milton-B was induced or not induced (p<0.01, N=4, Fig. 7E). Therefore, the retrograde redistribution induced by acute Milton-B OE of mitoGFP-tagged mitochondria is consistent with the hypothesis that Milton-B predominantly facilitates dynein-based retrograde transport of mitochondria in axons.

**Acute overexpression of Milton-B in sensory neurons induces a redistribution of mitochondria into secondary dendritic branches**

The depletion of mitochondria at NMJs and accumulation in the soma of the unipolar motor neurons in Milton-B OE animals (Fig. 7A, C-D) is consistent with an increase in dynein-driven mitochondrial transport. However, the accumulation of mitochondria at a single spot in the neuropil is difficult to interpret, as the orientation of microtubules in the primary neurite are not known (Fig. 5C; Stone et al., 2008). The complexity of the neuropil also makes it difficult to precisely localize mitochondria to axon-dendrite branch points, which cause mitochondrial accumulations seen in Milton-B OE animals (Fig. 6, 7A, arrows).

To bypass these constraints, we turned to multi-dendritic sensory neurons (SNs), specifically to class I vpda neurons (Grueber et al., 2002). The vpda neurons possess a distinct and clearly delineated dendritic arbor, in which a primary dendrite (Fig. 5D, 8A arrowheads)
branches out into parallel and coplanar secondary dendrites (Fig. 8A arrows). In contrast to mammalian dendrites, both primary and secondary dendrites exhibit a MT minus end-out orientation and require dynein for anterograde transport of mitochondria (Fig. 5D; Grueber et al., 2002; Rolls et al., 2007; Stone et al., 2008; Rolls, 2011).

Acute expression of mitoGFP for 24 hours in wild type vpda neurons reliably visualized mitoGFP-tagged mitochondria in irregularly spaced intervals in primary and secondary dendrites (Fig. 8A). Acute overexpression of Milton-B caused a significant redistribution of mitochondria within the dendritic arbor (Fig. 8A-B). Specifically, Milton-B OE significantly reduced the number of individual and clustered mitochondria in the primary neurite (p<0.001, n=6, Fig. 8D), while having no effect on the overall number of mitochondria in secondary dendrites (p>0.99, n=6, Fig. 8E). However, Milton-B OE significantly increased the average size of mitochondria in secondary dendrites (p<0.001, n=4, Fig. 8F). In wild type, the majority of mitoGFP positive punctae had an area of ~0.5 µm², indicating that most of them represent single mitochondria (Fig. 8G). In contrast, the frequency distribution of mitoGFP positive punctae in secondary dendrites of Milt B OE vpda neurons exhibited two peaks, one at 0.5 µm² (like wild type) and a second at ~2 µm² (Fig. 8G), indicating an increased amount of clustered mitochondria. These results indicate that Milton-B OE induces an increase of mitochondrial transport from the soma and the primary dendrite towards the minus-ends of dendritic microtubules, further supporting the hypothesis that Milton-B facilitates dynein-based mitochondrial transport.
Milton requires dMiro and its N-terminal GTPase activity to facilitate mitochondrial transport

Recent work in our lab demonstrated that dMiro facilitates both kinesin- and dynein-driven transport in vpda neurons, since both dendrites and axons of dmiro null mutants are essentially depleted of mitochondria (Chapter 2). In addition, inactivation of dMiro's N-terminal...
GTPase domain prevented both kinesin- and dynein-driven transport to a similar degree as \textit{dmiro} null mutations (Chapter 2). Previous cell culture studies, however, have shown that co-overexpression of kinesin (KHC) and Milton causes association of both proteins with mitochondria, as well as mitochondrial aggregation and redistribution towards the periphery of the cell (Glater, 2006). This association did not require co-overexpression of Miro, raising the possibility that Milton-KHC complexes may facilitate mitochondrial transport in a Miro-independent manner. On the other hand, mammalian studies have shown that overexpressed TRAK2 associates with mitochondria only when co-overexpressed with Miro1 (MacAskill et al., 2009a).

To determine whether Miro is essential for Milton-facilitated mitochondrial transport, we took advantage of the GeneSwitch drivers 176- and 734-Gal4 to induce expression of Milton-A/B in \textit{dmiro} null animals. Acute 24 hour expression of wildtype dMiro in 96-hour-old dmiro null mutant larvae partially restored the distribution of mitochondria into axons and dendrites (Fig. 8A-D). In comparison, overexpression of Milton-A alone in \textit{dmiro} null mutants had no effect; axons and dendrites (not shown) were still depleted of mitochondria to a similar degree as in \textit{dmiro} null mutants without Milton-A OE (p>0.99, N>5, Fig. 9A,C). Similarly, acute (24 hours) overexpression of Milton-B in \textit{dmiro} null mutants was unable to re-distribute mitochondria into axons and dendrites of sensory neurons (p>0.98, N=4, Fig. 9B,D), much in contrast to the effects of OE in wild type (Fig. 8). Together, these results suggest that dMiro is essential for Milton-A and -B facilitation of kinesin- and dynein-mediated transport, respectively.

To test whether Milton function requires dMiro only as a structural membrane-anchor, or also requires dMiro’s N-terminal GTPase domain activity, we co-expressed dMiroT25N, Milton-A and mitoGFP in \textit{dmiro} null mutant MNs. As reported earlier (Chapter 2), expression of dMiroT25N alone failed to restore the null mutant phenotype in axons as well as dendrites
The same failure of rescue was seen in the acute co-expression of dMiroT25N and Milton-A (p>0.99, N>5, Fig. 9A,C), while co-expression of normal dMiro with Milton-A partially rescued the mitochondrial depletion of axons (p<0.001, N>5, Fig. 9A,C).

Similarly, acute co-expression of dMiroT25N with Milton-B in dmiro null mutant SNs had no effect on the mitochondrial depletion of dendrites (p>0.94, n=6, Fig. 9B,D). In contrast, co-expression of normal dMiro with Milton-B in dmiro null animals increased the number of dendritic mitochondria significantly further than dMiro expression alone (p<0.01, n=6, Fig. 9A,D). In addition, when Milton-B was co-expressed in dmiro nulls with normal dMiro for 24 hours, it even caused a clustering of mitochondria in secondary dendrites (p<0.01, n=4, Fig. 9B,E), reminiscent of the phenotype observed with Milton-B OE in wildtype animals (Fig. 8F-G). Together, these results suggest that dMiro’s N-terminal GTPase activity is essential for Milton-A and -B facilitation of kinesin- and dynein-mediated transport, respectively.

Taken together, our analysis indicates that dMiro is the only transport-competent mitochondrial anchor for both Milton-A/kinesin and Milton-B/dynein motor complexes in
Drosophila. In addition, Milton also requires the activity of dMiro's N-terminal GTPase domain to facilitate mitochondrial transport.

Milton-B acts downstream of dMiro

Overexpression (OE) of dMiro (chronic or induced) in otherwise wild type Drosophila larval motor neurons leads to an accumulation of mitochondria in the terminal boutons of NMJs (Fig. 5A-B) but causes a depletion of mitochondria in dendrites (Chapter 2). A similar effect is seen in SNs in response to dMiro OE (Chapter 2). These effects are consistent with the idea that elevated levels of dMiro primarily recruit Milton-A/kinesin motor complexes to mitochondria, while Milton-B/dynein motor complexes may be outcompeted.

To test this further, we induced an acute 24 hour co-overexpression of both dMiro and Milton-B in otherwise wild type motor neurons, and compared the effects on the mitochondrial distribution in axons to those of the individual expression of each protein. Co-overexpression of dMiro and Milton-B depleted NMJ of mitochondria in manner that was indistinguishable from the from Milton-B overexpression alone (p>0.99, n>12, Fig. 10A-B). In addition, co-overexpression of Milton-B fully reverted the significant accumulation of mitochondria at terminal boutons induced by dMiro OE to an almost complete depletion (p<0.001, n>12, Fig. 10A-B).

Together these data show that Milton-B co-overexpression is able to fully suppress the effects of dMiro OE, indicating that dMiro acts upstream of Milton-B. In addition, these data suggest that dMiro, other than the transport-switching activity of its N-terminal GTPase domain, mostly acts as a membrane anchor for Milton-A/B motor complexes - without controlling the type of the motor complex. This is consistent with our previous suggestion that dMiro's N-terminal GTPase domain controls the transition from a stationary to motile state irrespective of the direction of transport (Chapter 2).
3.5 Discussion

Possibly the weakest point of neuronal cell biology is its reliance on long-distance cargo transport, which is required for their normal function, health and survival. A particularly pertinent example is mitochondrial transport, a mechanism required not just for maintenance of energy supply in distant compartments of neuronal cells, but also for mitochondrial quality control, mitochondrial biogenesis, general lipid synthesis and for many other aspects of normal cellular physiology (Goldstein, 2001; De Vos et al., 2008; Martin, 2012; Sheng and Cai, 2012). Despite its obvious importance, our understanding of the mechanisms which control and regulate mitochondrial transport have been sadly lacking.

Previous work has demonstrated that the balance of activity by the opposite-polarity MT motors kinesin and dynein determines the ultimate direction of long-distance mitochondrial transport, and therefore the subcellular mitochondrial distribution (Boldogh and Pon, 2006; Saxton and Hollenbeck, 2012). While the critical significance of the Miro-Milton complex for kinesin-mediated mitochondrial transport has been long known (Guo et al., 2005a; Glater et al., 2006; Russo et al., 2009b; reviewed in Lee and Lu, 2014), the interaction between Miro and dynein has remained obscure. In their study, van Spronsen and colleagues demonstrated that kinesin binding to Miro is actually mostly facilitated by TRAK1 in its extended conformation, while TRAK2 or TRAK1 in a folded conformation predominantly facilitate binding of dynein (van Spronsen et al., 2013).

Our analysis shows that the differential adaptor-motor binding mechanism of mitochondrial transport is conserved in flies, acting through alternately spliced isoforms of Drosophila Milton. Milton-A facilitates kinesin-based redistribution of mitochondria in axons, while Milton-B facilitates dynein-based redistribution in axons and dendrites. Thus, differential
binding by alternate adaptor-motor complexes can be added to a long list of mitochondrial transport functions which are extremely well conserved (Chapter 2; also see Chapter 4).

Curiously, while Milton-A OE did enhance kinesin-based mitochondrial transport, it also caused a massive mitochondrial degeneration phenotype. In contrast, Milton-B overexpression appeared to have a straightforward effect of increasing dynein-based mitochondrial transport, without showing any measurable negative effects on mitochondrial health. The Milton-A mitochondrial destruction mechanism remains mysterious, although it appears it may be linked to mitochondrial transport since expression of Milton-A in dmiro null animals fails to produce the cytosolic mitoGFP phenotype. However, if a transport malfunction is the cause, it remains unclear why it would be limited to kinesin-facilitating Milton-A, and not also seen in Milton-B overexpression.

We further show that both Milton isoforms require Miro as the only transport-competent mitochondrial anchor, resolving a long standing contradiction between opposing cell culture studies (Glater et al., 2006; MacAskill et al., 2009a). Furthermore, Miro was not sufficient just as a structural anchor; instead, the activity of its N-terminal GTPase domain was required to allow transport for both Milton-A and Milton-B. This result agrees with the findings of our previous study (Chapter 2), which demonstrated that Miro's N-terminal GTPase domain likely acts as a signaling switch controlling the transition from a stationary to a motile state.

Our previous study of dmiro null phenotypes (Russo et al., 2009b) made a curious observation: the null animals still occasionally exhibited very few highly abnormal motile mitochondria. While very small and rare, these mitochondria permitted analysis of motor kinetics in absence of Miro. Importantly, these mitochondria still appeared to have a preferred direction of transport, mostly moving either towards the soma or towards the synapse; the movements were impaired, and the mitochondria spent much more time stopped, but a clear preference for
either kinesin or dynein movements was measurable. This result strongly indicated that Miro is not the "deciding factor" in the determination of transport direction.

The same study demonstrated that overexpression of dMiro causes accumulation of mitochondria at the terminal boutons of the NMJ. When our current results are taken into consideration, the most likely explanation for this observation is that Miro has a preference for Milton-A/kinesin complexes (Russo et al., 2009b).

Our observation that Milton-B OE suppresses the dMiro OE phenotype provides an important additional clue. Namely, if Miro was somehow in a locked-in "kinesin-binding" mode within the axons of motor neurons, overexpression of Milton-B should have no effect on mitochondrial distribution, or, at the very least, the two phenotypes should compete with each other. Instead, we observed that coexpression of Milton-B and dMiro fully suppressed the dMiro OE-induced phenotype, such that the NMJ was devoid of mitochondria just as see with Milton-B OE on its own. This finding further suggests that Miro is likely not controlling the direction of transport.
CHAPTER 4: SUMMARY AND FUTURE DIRECTIONS

I have shown that Miro's G1 domain acts as an on/off switch for mitochondrial motility in general, irrespective of the motor used (Chapter 2). This mobilization activity is in line with the function of Miro's EF-hand domains, which are required for Ca\textsuperscript{2+}-mediated arrest of mitochondrial transport (MacAskill et al., 2009b; Wang and Schwarz, 2009). Curiously, the mobilization activity is reminiscent of Miro's function in yeast, where it undocks mitochondria from their association with ER, freeing them to move away from sites of ER-mediated fission (Murley et al., 2013).

Our results indicate that prevailing ideas about the function of Miro may need to be revised. For a long time, Miro has been seen as performing significantly different functions in metazoans and in yeast. This view has persisted despite accumulating evidence that practically all aspects of its structure and function are well conserved.

Our results indicate that Miro may perform a similar function in all eukaryotes, as a mediator of undocking mitochondria from the ER. In yeast, this function is limited to mitochondria-ER interactions; whereas in metazoans, it has been extended to MT-based transport, co-opting EF-hand and G1 activity for control of MT-based mitochondrial transport and distribution.

Furthermore, the results presented in Chapter 3 extend the differential adaptor-motor binding model of TRAK protein (van Spronsen et al., 2013) from mammals to flies. In addition, I further demonstrate that Milton activity critically depends on Miro and its G1 domain activity. Cumulatively, a revised model for Miro-driven mitochondrial transport in neurons is proposed.

Miro serves three main functions for mitochondrial transport. First, it couples motor adaptor complexes to mitochondria. Second, it promotes the transition from a stationary to a...
motile state through its N-terminal GTPase domain. Third, it stops mitochondrial transport through Ca\(^{2+}\) activation of its EF-hand motifs. Beyond these three clearly delineated roles, Miro may not significantly affect mitochondrial transport \textit{per se}, although its other unknown functionalities may modulate transport through secondary effects.

These three roles present a unified model, which most likely relies on mitochondria-ER contact sites. ER contact sites are known to contain IP3 receptors, which are capable of producing local Ca\(^{2+}\) microdomains (Szabadkai et al., 2006; Csordas et al., 2010). Previous studies have also shown that binding of Ca\(^{2+}\) by Miro's EF-hands may either dissociate motors from the complex (MacAskill et al., 2009b) or prevent the interaction of motors with MTs (Wang and Schwarz, 2009). In either case, a "docked" mitochondrion would have its motors inactivated by the local IP3R-mediated Ca\(^{2+}\) microdomain. When Miro's G1 domain resolves the docking site, the complex can drift away from the local Ca\(^{2+}\) current, allowing the motors to resume their normal function.

In this model, the direction of transport (after undocking) would be regulated by the choice of which adaptor-motor protein complex is bound to the Miro anchor; this choice, the data seems clear, is \textit{not} generated or transduced by Miro itself.

Of course, significant amount of work remains to be done. The most pressing question, of course, is that of the mechanism which chooses the direction of transport. We have many candidates which could regulate parts of the process (NGF signaling, the Abl kinase pathway, local binding of factors like DISC-1, and many others), but the exact manner in which all of these pieces come together remains mysterious.
APPENDIX A

(Journal of Neuroscience, 35(14):5754-5771)

Miro’s N-Terminal GTPase Domain Is Required for Transport of Mitochondria into Axons and Dendrites

Milos Babic,1,4* Gary J. Russo,1,3* Andrea Wellington,1 Ryan Sangston,1,5 Migdalia Gonzalez,2 and Konrad E. Zinsmaier1,2

1Department of Neuroscience, 2Department of Molecular and Cellular Biology, 3Graduate Program in Biochemistry and Molecular & Cellular Biology, 4Graduate Interdisciplinary Program in Neuroscience, and 5Undergraduate Program in Neuroscience and Cognitive Science, University of Arizona, Tucson, Arizona 85721

Received March 14, 2014; revised Jan. 11, 2015; accepted March 2, 2015.

Author contributions: K.E.Z. designed research; M.B., G.J.R., A.W., R.S., and M.G. performed research; M.B. contributed unpublished reagents/analytic tools; M.B., G.J.R., A.W., R.S., M.G., and K.E.Z. analyzed data; M.B. and K.E.Z. wrote the paper.

This work was supported by a grant to K.E.Z. from the National Institute of Neurological Disorders and Stroke (R01NS052664). G.J.R. has been supported by a National Institutes of Health training grant (T32 AG007434). The anti-tubulin antibody developed by Michael Klymkowsky, the anti-Brp antibody developed by Alois Hofbauer and Erich Buchner, and the anti-CSP antibody developed by K.E.Z. and Seymour Benzer were obtained from the Developmental Studies Hybridoma Bank, created by the National Institute of Child Health and Human Development of the National Institutes of Health and maintained at the University of Iowa, Department of Biology, Iowa City, IA 52242.

We thank Kathryn Cruice and Hojin Seo for technical help and Elliot Imler for critical comments on the manuscript.

*M.B. and G.J.R. contributed equally to this work.

The authors declare no competing financial interests.

Correspondence should be addressed to Konrad E. Zinsmaier, University of Arizona, Department of Neuroscience, Gould-Simpson Building 627, P.O. Box 210077, 1040 E. 4th Street, Tucson, AZ 85721-0077. E-mail: kez4@email.arizona.edu.

DOI:10.1523/JNEUROSCI.1035-14.2015

Copyright © 2015 the authors
A.1 Abstract

Mitochondria are dynamically transported in and out of neuronal processes to maintain neuronal excitability and synaptic function. In higher eukaryotes, the mitochondrial GTPase Miro binds Milton/TRAK adaptor proteins linking microtubule (MT) motors to mitochondria. Here we show that *Drosophila* Miro (dMiro) is critically required for the dynein-driven distribution of mitochondria into dendrites, besides the previously shown requirement for kinesin-driven axonal transport. In addition, we used the loss of function mutations dMiroT25N and dMiroT460N to determine the significance of dMiro’s N- and C-terminal GTPase domains, respectively. Expression of dMiroT25N in the absence of endogenous dMiro caused premature lethality and arrested development at a pupal stage. dMiroT25N accumulated mitochondria in the soma of larval motor and sensory neurons, and prevented their kinesin- and dynein-dependent distribution into axons and dendrites, respectively. dMiroT25N-mutant mitochondria were severely fragmented and exhibit reduced kinesin and dynein motility in axons. In contrast, dMiroT460N did not impair viability, mitochondrial size or the distribution of mitochondria. However, dMiroT460N reduced dynein motility during retrograde mitochondrial transport in axons. Finally, we show that substitutions analogous to the constitutively active Ras-G12V mutation in dMiro’s N- and C-terminal GTPase domains cause neomorphic phenotypic effects that are likely unrelated to the normal function of each GTPase domain. Overall, our analysis indicates that dMiro’s N-terminal GTPase domain is critically required for viability, mitochondrial size, and the distribution of mitochondria out of the neuronal soma irrespective of the employed motor, likely by promoting the transition from a stationary to a motile state.
A.2 Introduction

Transport of mitochondria into axons and dendrites, mediated by the microtubule (MT) motors kinesin and dynein, is critical for sustaining neuronal excitability, synaptic transmission, and survival. Impairments of mitochondrial transport contribute to a number of neurodegenerative disorders (reviewed by Mattson et al., 2008; Zinsmaier et al., 2009; Saxton and Hollenbeck, 2012; Sheng and Cai, 2012; Millecamps and Julien, 2013). However, mechanisms regulating mitochondrial motility out of the neuronal soma remain enigmatic.

The atypical GTPase Miro is an outer mitochondrial membrane protein found in almost all eukaryotes and typically exhibits two GTPase domains and two Ca$^{2+}$ binding EF-hand domains (Vlahou et al., 2011). Despite this evolutionary conservation, divergent roles have been suggested for Miro in lower and higher eukaryotes. In yeast, Miro is primarily required for mitochondrial homeostasis, facilitating the dissociation of mitochondria from the endoplasmic reticulum (ER) after ER-associated mitochondrial division and phospholipid exchange. In metazoa, Miro is primarily required for mitochondrial transport, although it also has been implicated in mitochondrial homeostasis (reviewed by Lee and Lu, 2014).

In flies, Drosophila Miro (dMiro) and Milton are both critically required for transport of mitochondria into axons (Stowers et al., 2002; Guo et al., 2005a; Russo et al., 2009b). Mechanistically, dMiro recruits cytosolic Milton, which serves as an adaptor linking the heavy chain of conventional kinesin (KHC) to mitochondria (Stowers et al., 2002; Glater et al., 2006). However, whether dMiro and Milton are required for dynein-mediated transport into dendrites remains unclear.

In mammals, mitochondrial motor linkage is mediated by two Miro genes, Miro1 and 2, and two Milton-like adaptor genes, trafficking protein kinesin binding 1 and 2 (TRAK; i.e., Milton-1/OIP106 and Milton-2/GRIF-1, respectively) (Fransson et al., 2003; Brickley et al., 2005;
Fransson et al., 2006; Koutsopoulos et al., 2010). TRAK1 is preferentially localized to axons, facilitating kinesin-mediated anterograde mitochondrial transport, whereas TRAK2 is abundant in dendrites, facilitating dynein-mediated anterograde transport (van Spronsen et al., 2013). Miro’s EF-hand domains control the activity of the Milton/TRAK-motor complex. In Drosophila, Ca\(^{2+}\) activation of the EF-hands triggers binding of Miro kinesin’s motor domain, disengaging kinesin from MTs while in mammals it triggers the release of kinesin from the Miro/TRAK complex (MacAskill et al., 2009b; Wang and Schwarz, 2009).

The role of Miro’s two GTPase domains for mitochondrial transport has been less clear. Biochemical studies showed that loss of Miro’s N- or C-terminal GTPase activity has no effect on the ability of Miro1 and 2 to coimmunoprecipitate TRAK proteins (Fransson et al., 2006). Loss of Miro1’s N-terminal GTPase activity also had no effect on the mitochondrial recruitment of TRAK2 (MacAskill et al., 2009a). However, a presumed constitutively active mutation of the N-terminal GTPase domain abolished the recruitment of TRAK2 in hippocampal cell cultures and altered the mitochondrial distribution (MacAskill et al., 2009a).

We performed a genetic analysis of analogous mutations in dMiro’s two GTPase domains. Our study suggests that only dMiro’s N-terminal GTPase domain is critically required for viability, mitochondrial size and the kinesin- and dynein-dependent distribution of mitochondria into axons and dendrites, respectively.

A.3 Materials & Methods

Fly Stocks

Flies were raised on standard cornmeal medium with dry yeast at 24°C. The strain expressing green fluorescent protein (GFP) tagged by an N-terminal mitochondrial localization signal (\(w^{118}\), \(P[w^{+}; UAS-mGFP]\)) was obtained from W. Saxton (University of California Santa Cruz, Santa
Cruz, CA). The point mutations \textit{dmiro}^{SD32} (29 bp deletion at Y89) and \textit{dmiro}^{B682} (W105/stop) prematurely truncate dMiro within the N-terminal GTPase domain and are considered null mutations (Guo et al., 2005a; Russo et al., 2009b). The motor Gal4 drivers OK6 (Aberle et al., 2002) and elav-C155 (Lin and Goodman, 1994) were used to express all UAS-transgenes in a wild type (\textit{w}^{1118}) or a hetero-allelic \textit{dmiro} null mutant genetic background (\textit{dmiro}^{B682/SD32}). For expression in sensory neurons, we used the Gal4 drivers 21-7, C380, 477, 2-21 and 5-40 (Grueber et al., 2003; Song et al., 2007; Sanyal, 2009).

For all experiments, strains containing a homozygous UAS-dMiro transgene in a wild type or heterozygous \textit{dmiro} null mutant genetic background (\textit{w}^{1118}; \textit{P}[\textit{w}^*, \textit{UAS-dMiro-X}] or \textit{w}^{1118}; \textit{P}[\textit{w}^*, \textit{UAS-dMiro-X}], \textit{dmiro}^{B682/TM6TbSb}) were crossed to strains containing a homozygous Gal4-driver and a UAS-mGFP transgene in a wild type or heterozygous \textit{dmiro} null background (\textit{w}^{1118}; \textit{P}[\textit{w}^*, \textit{Gal4-driver}]; \textit{P}[\textit{w}^*, \textit{UAS-mGFP}] or \textit{w}^{1118}, \textit{P}[\textit{w}^*, \textit{UAS-mGFP}], \textit{dmiro}^{sd32/TM6TbSb}) to generate progeny that express a single copy of the UAS-transgene driven by the Gal4 driver in the respective genetic background (\textit{w}^{1118}; \textit{P}[\textit{w}^*, \textit{Gal4-driver}]/\textit{P}[\textit{w}^*, \textit{UAS-dMiro-X}]; \textit{P}[\textit{w}^*, \textit{UAS-mGFP}]/+ or \textit{w}^{1118}, \textit{P}[\textit{w}^*, \textit{Gal4-driver}]/\textit{P}[\textit{w}^*, \textit{UAS-dMiro-X}] ; \textit{dmiro}^{sd32}, \textit{P}[\textit{w}^*, \textit{UAS-mGFP}]/\textit{dmiro}^{B682}).

**Generation of dMiro mutant transgenes**

The previously generated myc-tagged dMiro cDNA (dMiro-RC) (Guo et al., 2005a) was used as a template for site-directed mutagenesis (Stratagene QuikChange Mutagenesis) using standard procedures and the following primers: T25N, 5’

\textit{GCCGGGGTGGTGCAGCTCGTGTGATTCTGTCTCTG} 3’; A20V, 5’

\textit{GCTCGTCCGCGACGTCGGGTG} 3’; T460N, 5’
AAGGGATCAGGAAAGAATGGAATGTGCAGGGGATTC 3'; K455V, 5'
GTCATGTGATTGGACCAGTGGGATCAGGAAAGACTGG 3'.

After verifying the introduced point mutations by DNA sequencing, mutant cDNAs were directionally cloned into a pUAST vector cleaved with NotI and KpnI (Brand and Perrimon, 1993). Double mutants were generated by fusing cDNAs containing individual mutations in the N- and C-terminal GTPase domain. RsrlI and KpnI cDNA fragments were excised from pUAST-dMiroK455V and -T460N and inserted into RsrlI/KpnI cleaved pUAST-dMiroA20V and -T25N. pUAST plasmids were injected into w1118 embryos, generating transgenic animals (Rainbow Transgenic, Camarillo, CA). At least two independent strains were obtained for each transgene. After outcrossing all other chromosomes, strains containing homozygous UAS-transgenes on the 2nd chromosome were generated containing a wild type-like genetic background (w1118) or a dmiro null mutant background (dmiroB682).

To generate a transgene that expresses only a dMiro-RC transcript under the control of a dMiro promoter we excised the region including intron 3, the alternative spliced exon 4 and intron 5 from a copy of the dMiro genomic region. The excised region was then replaced in frame with the dMiroRC-specific exon 4. First, a genomic transgene was generated that contains the dmiro gene from 1975 bp upstream (chromosome 3R:24,039,705, flybase) to 3544 downstream (3R:24,034,186) of its major transcriptional start site (3R:24,037,730). The respective region was PCR amplified with the primers 5’ GCTGAGCCCGATCCCACACATCACC 3’ and 5’ CCTCGACAAGCCAGAGCTGTAGACTC 3’, and cloned into a pUAST vector (pUAST-dMiro). Next, 2 DNA fragments containing small overlaps were PCR amplified for recombineering. The first fragment contained the 3’ end of the dmiro gene, spanning from the 5’ end of exon 5 to the end of the previously cloned 3’ DNA dmiro gene fragment of pUAST-dMiro
(primers 5’ CCTGAGGCAATTCGGCCTGATGACGG 3’ (3R:24,035,001, 5’ end exon 5) and 5’ GTCACACCACAGAAGTTGTTCCCTC 3’ (in pUAST)). The second fragment was amplified from a dMiro-RC cDNA in the pUAST-myc-dMiro-RC vector, and contained half of exon 3 just including a unique RsrII site to the 5’ end of exon 5 (primers 5’ GAGCGGCCGATCGATTTGGCC 3’ (3R:24,036,143 within exon 3) and 5’ CCGTCATCAGCCGAATTGCCTCAGG 3’ (3R:24,035,001; 5’ end exon 5)). The 2 PCR fragments were then combined by recombineering with a RsrII/KpnI-cut pUAST-dMiro vector using a GeneArt seamless cloning kit (Invitrogen). After DNA sequence verification, the single-isoform version of genomic dMiro was PCR amplified with primers containing a 5’ NotI or KpnI site (5’ GAGCGGCCGCGCTGAGCCCGATCCCACACATCACC 3’ and 5’ GAGGTACCCCTCGACAAGCCAGCTGTGDTGCAGAC 3’), cleaved with NotI and KpnI and ligated into a NotI/KpnI-cut pBID vector (Wang et al., 2012). The pBID-genomic-dMiro-RC plasmid was injected into y¹, M(vas-int.Dm)ZH-2A w*; M(3xP3-RFP.attP’)ZH-22A embryos expressing φC31 integrase and containing an attP landing site on chromosome 2 at 22A (Bischof et al., 2007). Four independent lines were obtained and outcrossed to w¹¹¹⁸. Two lines were crossed into a dmiro null mutant background (dmiro⁸⁶⁸²).

**Generation of dMiro antibody**

A N-terminally His-tagged dMiro-RC cDNA fragment (residues 1-590, pET100 TOPO vector) was expressed in BL21 (DE3) E. coli cells. His-tagged dMiro was purified using Ni-TED agarose and standard denaturing conditions (Affymetrix, Cleveland, OH). The purified protein was injected into two guinea pigs using a standard 60 day injection protocol (Cocalico Biologicals, Reamstown, PA). The obtained antisera were screened by western blots using both fly head protein extracts and bacterially expressed peptides. One of the obtained antiserums showed a
positive signal and was retained. The specificity of the polyclonal serum GP5 was verified by the absence of a signal on western blots and immunostainings using dmiro null mutations.

**Western Blot Analysis**

Five to twelve larval brains were homogenized in 50 μl of PBS supplemented with 0.2% Triton-X (PBS-T) with protease inhibitors (Sigma). Equivalents of two larval brains were separated by 10% SDS-PAGE gel, transferred onto nitrocellulose membranes, and immunostained using primary antibodies in PBS-T at 4°C overnight, followed by a two-hour incubation with HRP-conjugated secondary antibodies at room temperature. Primary antibodies used were guinea pig anti-dMiro (1:50,000), mouse anti-β-tubulin (1:1000; Developmental Studies Hybridoma Bank, DSHB #E7, Research Resource Identifier (RRID) AB_2315513), and mouse anti-myc (1:1000; Cell Signaling Technology, #2276, RRID AB_2314825) in PBS 0.1% Tween, 5% nonfat dry milk. Secondary antibodies used are anti-guinea pig-HRP (1:20,000; Santa Cruz Biotechnology, Cat# sc-2438 RRID:AB_650492) and anti-mouse-HRP (1:20,000; Pierce, Cat# 32430 RRID:AB_1185566). Blots were imaged using ChemiDoc XRS (BioRad, Hercules, CA).

**Immunostainings**

Climbing third-instar Drosophila larvae were dissected in HL-6 solution and fixed in 4% paraformaldehyde (pH 7.3) for one hour or in Bouin’s solution for 3 min (for kinesin). After washing in PBS-T (PBS with 0.2% Triton X-100, pH 7.3) and blockinf with Superblock (Thermo Scientific) or 0.2 % BSA for one hour, the preparation was incubated with primary antibody overnight at 4°C, washed 3x in PBST for 10 minutes, and incubated with secondary antibody for 2 hours at room temperature. Confocal images were acquired within two hours after final washing. The following antibodies and dilutions were used: anti-HRP-Cy3 at 1:250 (Jackson
ImmunoResearch, #123-165-021); anti-GFP-AF488 at 1:500 (Molecular Probes, #A21311 RRID:AB_221477); mouse anti-myc at 1:3000 (Cell Signaling, #2276); anti-dynein at 1:250 (DHSB, #2C11-2-s); anti-KHC at 1:50,000 (Cytoskeleton, #AKIN010); mouse anti-CSP at 1:200 (DHSB, #DCSP-2 (6D6), RRID:AB_528183); mouse anti-Bruchpilot at 1:400 (DHSB, #NC-82, RRID:AB_528108); RRID:AB_10707921); anti-mouse-Cy3 at 1:500 (Jackson ImmunoResearch, #715-165-150); goat anti-mouse-AF488 at 1:500 (Invitrogen, #A21121, RRID: AB_10053811); goat anti-rabbit-Cy3 at 1:500 (Jackson ImmunoResearch, #111-165-003, RRID:AB_2307387) and goat anti-HRP-AF647 at 1:500 (Jackson ImmunoResearch, #123-605-021, RRID:AB_2338967).

To immunostain sensory neurons in the body wall, dissected larvae were fixed for 15 minutes in 4% paraformaldehyde (pH 7.3), washed in PBS, and incubated at 4°C overnight in a 1:1000 dilution of anti-GFP AlexaFluor488 and a 1:400 dilution of anti-HRP-Cy3 in PBS with 0.2% or 0.5% Triton X-100.

Analysis of mitochondrial transport in axons

Mitochondria were live imaged in motor neurons of dissected climbing third instar larvae using HL-6 solution supplemented with 7 mM L-glutamate and 0.6 mM CaCl₂, as described previously (Louie et al., 2008; Russo et al., 2009b). Briefly, images of 1024x280 pixels (~58.9 x 16.1 μm) were acquired from individual segmental nerves near the ventral nerve cord. A centered region of interest (ROI, 872x278 pixels) was photobleached for 180 s followed by the immediate acquisition of 200 images (1024x280 pixels, one frame per 1.006 s). The movements of up to 12 clearly labeled mitochondria were tracked if they remained visible in at least 30 consecutive frames using NIH ImageJ imaging software (Schneider et al., 2012) and the plug-in MTrackJ (Meijering, E., University Medical Center of Rotterdam, Netherlands) as described previously.
(Louie et al., 2008). The obtained x–y–t coordinates of mitochondrial movements were analyzed with Java-based software that expands on the Excel-based analysis software described earlier (Louie et al., 2008). An individual movement was defined by a minimal displacement of 0.151 μm/s. Mitochondria undergoing antero- and retrograde transport were distinguished based on their overall net movement direction before calculating the described parameters as described previously (Russo et al., 2009b).

Quantification of motile mitochondrial densities in axons

Images 1024x280 pixels (~58.9 x 16.1 μm) of mGFP labeled mitochondria of larval motor axons were obtained at a rate of 1 s⁻¹, pseudo-colored in green (time = 0 sec (green) and magenta (time = 120 sec), and merged to visualize motile (green and magenta) and stationary (white) mitochondria. Since mitochondria marked green in the merged images were transported out of the ROI during the imaging period and magenta-marked mitochondria were transported into the ROI, only green- and white marked mitochondria were used to determine the density of motile and stationary mitochondria at time = 0. A mitochondrion that was displaced by at least three-quarters of its length was considered to be motile. The number of mitochondria per ROI was normalized to the respective area. The percent of motile mitochondria was determined as a fraction of the total number of mitochondria in a nerve ROI.

Quantification of mitochondrial densities

To determine the mitochondrial density at neuromuscular junctions (NMJs) of larval motor neurons (MNs) and dendrites of sensory neurons (SNs), respective confocal images were obtained from dissected 3rd instar larvae that were immunostained for mGFP-labeled mitochondria and neuronal membranes (anti-HRP). For analysis of NMJs, images were
obtained from muscles 6/7 in segment 3-4. The area of a synaptic bouton marked by HRP and the respective area of the bouton occupied by mitochondria marked by mitoGFP was measured using ImageJ software to determine the fraction of the synaptic area occupied by mitochondria. For analysis of dendrites, images of ventral posterior da (vpda) sensory neurons were obtained from segment A2. The length of primary and secondary dendrites and the number of mitochondria in the respective dendrites was measured using ImageJ software. The amount of dendritic mitochondria was normalized to dendritic length.

**TMRM Imaging**

3rd instar larvae were dissected in HL-6 solution supplemented with 7 mM L-glutamic acid in the absence of Ca$^{2+}$. Larvae were incubated for 25 minutes in HL-6 solution supplemented with 20nM Tetramethylrhodamine methyl ester perchlorate (TMRM, InVitrogen), rinsed briefly, and incubated for imaging in HL-6 solution containing 5 nM TMRM. Mitochondria at larval NMJs were imaged using a confocal microscope equipped with a multi-argon 488 laser to detect mGFP and a HeNe 543 laser to detect TMRM. To normalize the TMRM signal, mitochondria were then depolarized using 3 μM Carbonyl cyanide 3-chlorophenylhydrazone (CCCP, Sigma) in HL-6 solution containing 5 nM TMRM. After 3 minutes, images of the depolarized mitochondria were obtained using the red and green channels. ImageJ imaging software was used to measure the mean TMRM fluorescence intensity of presynaptic mitochondria in single slices before and after CCCP treatment. TMRM fluorescence was expressed as $\Delta F = (F_{\text{before CCCP}} - F_{\text{after CCCP}})/ F_{\text{before CCCP}}$. 
Statistical Analysis

All data with a non-Gaussian distribution are presented by box-whisker plots showing the median (line), the 25th–75th percentile (box), and the 10th–90th percentile (whisker) ranges. Data with a Gaussian distribution are presented by graph bars showing the mean ± SEM. Data were evaluated for their statistical significance by Mann-Whitney, one-, or two-way ANOVA testing and the indicated post-hoc tests using Prism software (Graphpad Software, San Diego California USA, www.graphpad.com). N and n indicate number of animals and mitochondria, respectively. P values of <0.05, <0.01, and <0.001 are indicated with one, two and three asterisks, respectively.
A.4 Results

Our previous studies established that dMiro is critically required for the axonal transport of mitochondria into larval motor neurons (MNs) of *Drosophila* (Guo et al., 2005a; Russo et al., 2009b). To determine whether this function requires the activity of dMiro's two GTPase domains, we generated dMiro cDNA-transgenes containing established loss as well as potential gain of function mutations using the Gal4 system for targeted gene expression (Brand and Perrimon, 1993). To abolish GTPase activity, we introduced the amino acid substitutions T25N in the N-terminal and T460N in the C-terminal GTPase domain of dMiro (Fig. A1A). Analogous substitutions were previously used in yeast (S19N and S462N) and mammalian Miro (N18 and N432). In yeast, S19N and S462N abolished GTP hydrolysis and failed to rescue mitochondrial defects of yeast Miro deletion mutants (Kornmann et al., 2011; Koshiba et al., 2011; Murley et al., 2013). In mammals, the substitutions N18 and N432 were regarded as dominant-negative mutations (Fransson et al., 2003; Fransson et al., 2006; MacAskill et al., 2009a) due to their similarity to the dominant-negative Ras-S17N mutation (Feig and Cooper, 1988; Feig, 1999; Cantrell, 2002). However, yeast MiroS19N and -S462N did not induce dominant phenotypes (Koshiba et al., 2011).

We also introduced the amino acid substitutions A20V and K455V into dMiro's N- and C-terminal GTPase domain, respectively. Ectopic expression of analogous mutations in human Miro1 and 2 (13V and 427V) altered the structure and distribution of mitochondria in cultured cells. These dominant effects were suggested to be of a constitutively active nature (Fransson et al., 2003; Fransson et al., 2006; Saotome et al., 2008; MacAskill et al., 2009a) since both substitutions were modeled after the constitutively active Ras-G12V mutation (Wittinghofer, 1998). However, since the two respective amino acids are divergent among human Miro, yeast Miro, and dMiro (Fig. A1A), the genetic nature of the mutations required further studies.
Figure A1. Transgenic expression of GTPase-mutant dMiro proteins. (A), amino acid sequence of the P loop in the N- (upper) and C-terminal GTPase domain (lower) of human Miro1 (hMiro1), yeast Miro (pGem1), and dMiro. The point mutations (T25N, T460N, A20V, K455V) introduced into the GTPase domains of myc-tagged dMiro (RC-transcript, FlyBase) are highlighted in red. (B-C), normal myc-dMiro (control) and mutant myc-dMiroA20V, -T25N, -K455V, and -T460N were pan-neuronally expressed in dmiro null mutants (-/-, B) or (C) overexpressed (OE) in otherwise wild type animals (w1118) using an elav-Gal4 driver. Viability was assayed daily by counting freshly hatched adult flies. Data represent means ± SEM of three independent matings. Only significant differences between control and mutant genotypes are indicated by asterisks (p<0.05, N>3, n>100, one-way ANOVA, Tukey’s post test). (D-E), protein expression levels of myc-tagged GTPase-mutant dMiro proteins that were transgenically expressed in dmiro null mutant (-/-) neurons. Immunoblots of larval brain protein extracts were stained with anti-Myc (D) or anti-dMiro antibodies (E), β-tubulin was used as loading control to normalize mutant protein levels to control (dMiro, 1.0; dMiroA20V, 0.9; dMiroT25N, 0.8; dMiroK455V, 1.1; dMiroT460N, 0.9; n=3). Genotypes are indicated. (F), co-immunolocalization of the mitochondrial marker mitoGFP (green, top row) with myc-tagged dMiro proteins (red, middle row) in MN cell bodies of 3rd instar larvae. Wild type control (WT) neurons lack transgenic expression of myc-dMiro. Transgenes encoding dMiroA20V, -T25N, -K455V, -T460N and normal dMiro were co-expressed with a transgene encoding mitoGFP in motor neurons of dmiro null mutants with an Ok6-Gal4 driver. Scale bar, 2 µm.
To verify proper expression of the myc-tagged mutant proteins, we expressed the respective cDNA transgenes in neurons of heteroallelic *dmiro* B682/SD32 null mutant animals using the pan-neuronal elav-Gal4 driver. Expression levels of mutant dMiro proteins were comparable to levels of UAS-expressed normal dMiro (±0.2-fold of control, \( p > 0.05 \); Fig. A1D) but significantly higher than levels of endogenously expressed dMiro (Fig. A1D,E). Immunostainings using anti-myc antibodies confirmed a mitochondrial localization of the transgenically expressed GTPase-mutant dMiro proteins (Fig. A1F).

**dMiro’s N-terminal GTPase domain is critical for survival.**

An interesting aspect of dMiro’s role for mitochondrial biology is that only its neuronal function is critical for survival of the animal (Guo et al., 2005a; Russo et al., 2009b). To determine the significance of dMiro’s GTPase domains for viability, we compared the phenotypic effects of expressing normal (control) and GTPase-mutant dMiro in heteroallelic *dmiro* null mutant neurons (dmirosd32/B682, Guo et al., 2005a). This strategy allowed a dual assessment of the mutant protein in regard to the absence of endogenous dMiro activity (null) and its ability to restore normal function in comparison to normal dMiro. Thereby, this strategy minimized potential complications associated with dominant-negative and/or constitutively active mutations (Feig, 1999; Cantrell, 2002). To evaluate the potential dominant nature of the examined mutations, we also overexpressed normal and mutant dMiro in an otherwise wild type genetic background (w^{1118}).

In comparison to normal dMiro, elav-driven pan-neuronal expression of dMiroT25N in *dmiro* nulls was lethal during late 3rd instar and early pupal development and adult escapers were never observed (\( p < 0.001 \); Fig. A1B), suggesting that a normal activity of dMiro’s N-terminal GTPase domain is critical for survival. In contrast, neuronal overexpression (OE) of
dMiroT25N had no significant effect on viability (p=0.7, Fig. A1C), indicating that dMiroT25N might not be a dominant-negative mutation.

Expression of dMiroA20V in dmiro nulls reduced viability in comparison to normal dMiro (p<0.01, Fig. A1B). However, dMiroA20V allowed a limited number of adult survivors, which exhibited crumpled wings, progressive defects in locomotion and rarely survived longer than for a few days. dMiroA20V OE also significantly impaired viability (p<0.001, Fig. A1C) to similar levels as dMiroA20V expression in nulls (Fig. A1B-C). This dominant OE effect could not be easily reconciled with the expected effects of a constitutively active mutation since the lethality induced by dMiroA20V expression in nulls was not enhanced by the presence of endogenous dMiro.

Neuronal expression of dMiroT460N and dMiroK455V in dmiro nulls restored a normal viability (p>0.7, Fig. A1B), indicating a dispensable role of dMiro’s C-terminal GTPase domain. In addition, neuronal OE had no significant effects on viability (p>0.1, Fig. A1C).

**dMiro’s N-terminal GTPase domain is required for a normal mitochondrial distribution in axons.**

Null mutations of dMiro cause a severe depletion of mitochondria in axons and dendrites of larval MNs that is accompanied by an accumulation of mitochondria in the soma (Fig, 2A; (Guo et al., 2005a; Russo et al., 2009b)). To test whether the two GTPase domains of dMiro are required for a normal distribution of mitochondria, we co-expressed mitoGFP with mutant or normal dMiro protein (control) in larval MNs of heteroallelic dmiro null mutants using the Ok6-Gal4 driver. Expression of dMiroT25N failed to restore a normal subcellular distribution of mitochondria in MNs, leaving dendrites, axons, and NMJs depleted of mitochondria (p<0.001, Fig. A2B-G). Instead, mitochondria accumulated in the soma of MNs in the same way it
Figure A2. dMiro’s N-terminal GTPase domain is required for a normal distribution of mitochondria in motor neurons. (A-J), mitoGFP was transgenically co-expressed with normal myc-dMiro (control), myc-dMiroA20V, -T25N, -K455V, or -T460N in MNs of dmiro null mutants (Null) using an Ok6-Gal4 driver. Mitochondria in larval MNs were visualized by confocal live imaging (A, H-J) or immunostainings using anti-GFP and anti-HRP antibodies (B-G). Graph bars represent mean ± SEM. Box whisker plots represent median (line), 25th–75th percentile (box), and 10th–90th percentile (whisker) ranges. Significant differences among indicated genotypes are indicated by asterisks (one-way ANOVA and Tukey post test or Kruskal-Wallis and Dunn’s post test).

(Figure legend continued on next page.)
accumulated in the soma of MNs of dmiro-null mutants (Fig. A2A). The severe depletion of mitochondria at dMiroT25N mutant NMJs was similar to the effects of dmiro null mutations (p>0.05, Fig. A2E-G). Like dmiro null mutants, dMiroT25N-mutant synaptic boutons exhibited a significantly reduced size in comparison to control (p<0.001, dMiro 9.0±5.5 (SD), n=247; dMiroT25N 5.4±3.2, n=113; dmiro 6.9±5.8, n=113). Hence, a normal activity of dMiro’s N-terminal GTPase domain appears critical for a normal distribution of mitochondria into axons, which primarily requires kinesin-driven transport (Pilling et al., 2006).

The impaired dendritic distribution of mitochondria in the unipolar MNs of dMiroT25N and dmiro null mutants (Fig. A2A) is difficult to interpret since the MT organization in the primary neurite is not known, even though it has been resolved for axons and dendrites (Stone et al., 2008). Hence, the defect may be either caused by a primary defect in kinesin-driven transport while traversing the primary neurite, by a dendritic defect in dynein-driven transport, or by a combination of both.

In contrast to dMiroT25N, expression of dMiroT460N in dmiro null mutant MNs facilitated a normal distribution of mitochondria in the soma, proximal motor axon and the NMJ in comparison to dMiro control (p>0.05, Fig. A2A-G). Notably, expression of normal dMiro in dmiro nulls causes an accumulation of mitochondria in synaptic boutons that is especially pronounced at terminal boutons (Guo et al., 2005a; Russo et al., 2009b). dMiroT460N did not disrupt this
mitochondrial accumulation at terminal boutons (p=0.9; Fig. 2F-G) and the proportional increase in bouton area (not shown). Hence, a normal activity of dMiro’s C-terminal GTPase domain is likely expendable for the distribution of mitochondria into neuronal processes.

Expression of dMiroA20V and dMiroK455V in dmiro nulls facilitated a normal distribution of mitochondria in axons and NMJs. The mitochondrial density in proximal axons, the amount of synaptic boutons invaded by mitochondria and the mitochondrial area of synaptic boutons including terminal boutons was similar to control (p>0.05, Fig. A2B-G). dMiroA20V and dMiroK455V also had no effect on the average size of synaptic boutons in comparison to control (p>0.3, dMiro 9.0±5.5 (SD), n=247; dMiroA20V 11.2±8.1, n=169; dMiroK455V 9.4±5.1, n=60).

To assess potential effects on the distribution of other synaptic cargoes, we examined the synaptic distribution of vesicle-associated Cysteine-string protein (CSP) (Zinsmaier et al., 1994) and the active zone component Bruchpilot (Brp) (Wagh et al., 2006). The synaptic levels of CSP were reduced in synaptic boutons of dMiroT25N, -A20V, and dmiro null mutants from 78% to 84% of control (p<0.001) while dMiroT460N and dMiroK455V had no significant effect (p>0.7, Fig. A3A, D). The levels of Brp were decreased in synaptic boutons of dMiroT25N, -A20V -T460N and -K455V to various degrees ranging from 54% to 71% of control (p<0.001, Fig. A3B, E). In addition, the intensity of anti-HRP stained neuronal membranes was significantly reduced at NMJs of dMiroT25N, -T460N, -K455V and dmiro null mutants (p<0.001) while dMiroA20V had no significant effect (p=0.99, Fig. A3F).

Since anti-HRP antibodies detect at least 7 different neuronal proteins in Drosophila (Paschinger et al., 2009), the reduced HRP staining could indicate potential problems in mitochondrial energy supply or protein logistics. To test for a general axonal transport defect, we expressed normal and dMiro mutant proteins in all neurons to ensure a homogenous genotype of sensory and motor axons in the larval nerve. However, we found no evidence for
Figure A3. Effects of mutations in dMiro’s GTPase domains on the distribution of synaptic components. (A-H), normal (control) and GTPase mutant myc-dMiro proteins (A20V, T25N, K455V, or T460N) were expressed in dmicro null mutant (Null, /-) neurons using an elav-Gal4 driver. Neuronal membranes, synaptic vesicles and active zones at larval NMJs on muscle 6/7 were visualized by confocal imaging of immunostainings using anti-HRP, anti-CSP and anti-Brp antibodies, respectively. Asterisks indicate significant differences among indicated genotypes (Kruskal-Wallis, Dunn’s post test). (A-B), distribution of synaptic vesicle-associated CSP (A) and the active zone component Brp (B) at synaptic boutons of larval NMJs visualized by HRP stainings. Genotypes are indicated. Scale, 20 µm. (C), accumulations of CSP (green) and HRP (red) in sensory and motor axons of larval nerves proximal to the VNC. Scale, 10 µm. (D-F), quantification of CSP (D), Brp (E) and HRP (F) fluorescence at immunostained synaptic boutons of larval NMJs that were normalized to dMiro control levels. Genotypes are indicated (p<0.05, n>56, N>4). (G), Quantification of protein accumulations that were immunopositive for CSP and HRP in sensory and motor axons of larval nerves proximal to the VNC (p>0.05, N>10).
general traffic jams of axonal cargoes ($p>0.05$, Fig. A3G), which typically can be visualized by an accumulation of synaptic vesicle precursors and other cargo vesicles transporting neuronal proteins (Gunawardena and Goldstein, 2001; Gunawardena et al., 2003). Overall, the phenotypic effects of the GTPase mutations on neuronal proteins did not correlate with the effects on the axonal distribution of mitochondria. Accordingly, the severe depletion of mitochondria in dMiroT25N mutant axons is not caused by a general defect of the axonal transport machinery.

**Mutations in dMiro’s N- and C-terminal GTPase domains alter the structure of axonal mitochondria.**

A number of previous studies reported effects of altered Miro levels or mutations in Miro’s GTPase domains on mitochondrial structure from yeast to mammals (Frederick et al., 2004; Fransson et al., 2006; Saotome et al., 2008). In flies, *dmiro* null mutations also reduced the average length of axonal mitochondria to less than $\sim 0.6 \, \mu m$ (Russo et al., 2009b). dMiroT25N expression in *dmiro* null mutant MNs caused a similar effect and significantly reduced the length of both motile and stationary (not shown) mitochondria ($p<0.001$, Fig. A2B, H), suggesting a potential role of dMiro’s N-terminal GTPase domain for maintaining mitochondrial structure.

dMiroA20V, -K455V and -T460N had no significant effect on the length of motile mitochondria in axons ($p=0.99$, Fig. A2B, H). However, both dMiroA20V and dMiroK455V significantly increased the number of stationary mitochondria that exceeded a length of more than 6 $\mu m$ ($p<0.01$, Fig. A2B, I). In addition, both dMiroA20V and -K455V caused two morphologically different types of elongated axonal mitochondria: one type exhibited a normal width while the second type exhibited extraordinarily thin regions that protruded from sections of normal width and were often connected to other mitochondria of normal width (Fig. A2B,
Since this shape is reminiscent of a mitochondrion being pulled apart by opposing forces, it seems possible that this phenotype could be due to an impaired coordination of transport, causing a tug-of-war between kinesin and dynein motors. Alternatively, this abnormal shape could be caused by an impaired dissociation of stationary mitochondria from a “docking

Figure A4. dMiro’s N-terminal GTPase domain is required for normal motility of axonal mitochondria. (A-E), mitoGFP was co-expressed with myc-dMiro (control, dMiro, -/-), -A20V, -T25N, -K455V, or -T460N in MNs of dmiro null mutants (Null, +/-) using an Ok6-Gal4 driver. Confocal time-lapse images visualizing mitochondria in motor axons of the segmental larval nerve exiting the VNC were acquired at a rate of 1 s⁻¹. Asterisks indicate significant differences of indicated mutant genotypes from controls (Kruskal-Wallis and Dunn’s post test). (A), distribution of mitochondria that were motile (green or magenta) or stationary (white) in proximal axons of motor neurons during the 2 minute long imaging period. Scale bar, 5 µm. (B-C), overall density of motile (B) and stationary (C) mitochondria in proximal axons of motor neurons (p<0.05, N>7). (D-E), mitochondrial flux of antero- (D) and retrograde (E) transport in motor axons of proximal larval nerves (p<0.05, N>15).
site," or an impaired coordination of mitochondrial fission with transport. Since overexpression of dMiroA20V and -K455V had similar effects (not shown), an interpretation of these dominant neomorphic effects is difficult (see discussion).

Notably, both dMiroA20V and -K455V also induced the formation of abnormally large and swollen mitochondria, which could indicate a deterioration of mitochondrial structure due to a loss of mitochondrial health. To address this possibility, we measured the membrane potential of axonal mitochondria at larval NMJs using the cell-permeant potentiometric fluorescent dye tetramethylrhodamine methyl ester (TMRM) (Ehrenberg et al., 1988; Plasek and Sigler, 1996). In comparison to wild type control, expression of normal dMiro in dmiro mutants caused a significant increase in the average TMRM fluorescence of mitochondria (p<0.001, Fig. A2J), which is likely caused by the higher than normal expression levels of dMiro (Fig. A1D-E). Expression of dMiroT460N and -K455V caused a similar increase (p>0.8). However, dMiroA20V mutant mitochondria exhibited normal TMRM fluorescence levels in comparison to wild type (p=0.4, Fig. A2J). Hence, the structural effects on mitochondria induced by dMiroA20V and -K455V expression cannot be explained by a deteriorated mitochondrial membrane potential.

**Loss of dMiro’s N-terminal GTPase activity impairs both kinesin- and dynein-driven transport of axonal mitochondria.**

To directly examine the requirement of dMiro’s GTPase domains for axonal transport of mitochondria, we live-imaged mitoGFP-tagged mitochondria in proximal motor axons of larval
nerves, as previously described (Louie et al., 2008). This system allows for an unambiguous
distinction between kinesin- and dynein-driven movements (MT plus and minus end-directed,
respectively) because the axons exhibit a uniform MT plus end-out orientation (Pilling et al.,
2006; Stone et al., 2008). Of the few mitochondria present in dMiroT25N-mutant axons, most
were stationary for more than 2 minutes and only very few were motile in comparison to control
(p<0.001, Fig. A4A-C), consistent with a severely reduced flux (rate) of both anterogradely and
retrogradely moving mitochondria (p<0.001, Fig. A4D-E). However, the latter defect could
simply be a consequence of the severe depletion of axonal mitochondria.

To examine the effects of dMiroT25N on mitochondrial motility, we tracked the transport
of mitoGFP-tagged mitochondria in larval motor axons over a 200 second period (Fig. A5A).
Transport of mitochondria in a given direction is the net product of opposing movements that are
driven by the opposite polarity motors kinesin and dynein, which drive alternating bidirectional
movements that are interspersed with short stationary phases (Morris and Hollenbeck, 1993;
Hollenbeck, 1996; Russo et al., 2009b; Saxton and Hollenbeck, 2012). Individual movements of
a mitochondrion were termed a “run” (Morris and Hollenbeck, 1993; Hollenbeck, 1996; Pilling et
al., 2006; Russo et al., 2009b), which we further categorized into two types: individual runs
(iRuns), which are runs that are each preceded and followed by a run in the opposite direction;
and consecutive runs (cRuns), which are runs that are each preceded or followed by a run in
the same direction. Kinesin motors in particular use on average 4.2 cRuns before a reversal in
direction occurs. This phenomenon is described by the term ‘trip”, which is exclusively defined
by a reversal in transport direction, while a run is defined by either a stop or a reversal in
direction. Hence, “trips” can be formed by a single iRun or multiple cRuns.

The long-distance transport of mitochondria into both axons and dendrites is mediated
by the opposite-polarity microtubules (MT) motors kinesin and dynein, giving rise to alternating
Figure A5. Effects of dMiroT25N and dMiroT460N on kinesin-driven motility during anterograde axonal transport of mitochondria. (A-I), mitoGFP was co-expressed with myc-dMiro (control, dMiro, +/-), -T25N or -T460N in motor neurons of dmiro null mutants (Null (-/-)) using an Ok6-Gal4 driver. Confocal time-lapse images of mitoGFP-tagged mitochondria in motor axons in the segmental larval nerves exiting the VNC were acquired at a rate of 1 sec^{-1} immediately after photobleaching a 50-µm-long region of interest to track motions of mitochondria moving into the bleached area. Asterisks indicate significant differences among indicated genotypes (Kruskal-Wallis and Dunn’s post test). (A), each plot shows typical tracks of movements by individual mitochondrion for a duration of 180 s. For comparison, the start of individual tracks was set to zero. Net-positive and -negative movements represent antero- and retrograde transport, respectively. (B), net-velocity of anterograde mitochondrial transport in motor axons (p<0.001, N>20). (C-D), fraction of time mitochondria spent on MT plus end-directed trips (C) and short stops (D) during anterograde transport (p<0.05, N>20). (E), average duration of stops of mitochondria during anterograde transport (p<0.05, N>18). (F-G), distance of MT plus end-directed cRuns (F) and trips (G) during anterograde mitochondrial transport (p<0.001, N>20). (H), number of MT plus end-directed cRuns during anterograde mitochondrial transport (p<0.01, N>20). (I), fraction of MT plus end-directed trips that exhibited cRuns (p<0.05, N>29).
bidirectional movements that are interspersed with short stationary phases (Morris and Hollenbeck, 1993).

dMiroT25N severely impaired anterograde mitochondrial transport. In comparison to normal dMiro, dMiroT25N expression in dmiro nulls significantly reduced the net-velocity of anterograde transport (p<0.001, Fig. A5A-B), even though a few dMiroT25N-mutant mitochondria (~1 out of 20) exhibited relatively normal anterograde transport (Fig. A5A). Specifically, dMiroT25N reduced the time mitochondria spent on MT plus end-directed, kinesin-driven trips (p<0.001), the distance of kinesin-driven runs and trips (p<0.001), the number of kinesin-driven cRuns (p<0.001) and the percentage of kinesin trips containing more than one run (p<0.01, Fig. A5C, F-I). In addition, dMiroT25N significantly increased the duration of stops and the time mitochondria spent motionless (p<0.001, Fig. A5D-E). All of these effects induced by dMiroT25N were statistically indistinguishable from those of dmiro null mutations (p>0.05, Fig. A5B-I). The impaired kinesin motility of anterogradely moving mitochondria was not caused by a tug-of-war between kinesin and dynein motors since dMiroT25N had no significant effect on the distance of dynein-driven runs (ctrl 0.27±0.1 µm; T25N 0.28±0.14 µm; p=0.9, Mann-Whitney) and trips (ctrl 0.30±0.13 µm; T25N 0.35±0.16 µm; p=0.14). Taken together, the severe defects induced by dMiroT25N indicate a critical role of dMiro’s N-terminal GTPase domain for kinesin-mediated movements driving anterograde transport.

dMiroT25N affected retrograde mitochondrial transport less severely than anterograde transport. dMiroT25N expression in dmiro nulls impaired the net-velocity of retrogradely moving mitochondria to a level that was significantly different to both dmiro nulls and normal dMiro (p<0.05, Fig. A6A). In addition, dMiroT25N impaired the distance of dynein-mediated iRuns (not shown), cRuns and trips in comparison to control (p<0.05; Fig. A6E-F). Like dmiro null mutations, dMiroT25N also had no effect on the number of dynein-driven cRuns and the
percentage of dynein trips containing cRuns ($p>0.05$; Fig. A6G-H). In contrast to $dmiro$ nulls, dMiroT25N did not significantly affect the preferential use of MT dynein-driven trips, the time mitochondria spent motionless and the duration of stops ($p>0.05$; Fig. A6B-D). The differential effects of dMiroT25N and $dmiro$ null mutations on retrograde transport excluded the possibility that T25N disrupts all protein activities of dMiro.

Notably, a very small fraction of T25N-mutant mitochondria exhibited almost normal antero- and retrograde transport (Fig. A5A), which might be due to a maternal effect or a second form of mitochondrial motor linkage that is independent of dMiro, as it has been suggested for fly and mammalian systems (Glater et al., 2006; Koutsopoulos et al., 2010).

**Genetic nature of dMiroT25N.**

To evaluate the genetic nature of dMiroT25N, we overexpressed dMiroT25N in otherwise wild type-like larval MNs ($w^{118}$). In the case of a dominant-negative mutation, one expects that expression in a wild type and a null background would induce qualitatively similar phenotypes, even though increased levels of endogenous protein may gradually decrease the phenotypic severity of the mutation (Muller, 1932; Wilkie, 1994). Hence, one expects that dMiroT25N overexpression (OE) would deplete both dendrites and axons of mitochondria, like its expression in $dmiro$ nulls (Fig. A2A). However, this was not the case. dMiroT25N OE in MNs did not deplete axons of mitochondria (arrows, Fig. A7A). In contrast, dMiroT25N OE caused an accumulation of mitochondria at terminal boutons of NMJs in comparison to wild type ($p<0.001$), which was similar to the accumulation induced by normal dMiro OE ($p=0.52$; WT $3.9\pm1.6$ (SD); dMiroT25N OE $7.1\pm1.9$; dMiro OE $8.0\pm2.7$: $N>10$). However, dMiroT25N OE had no gross effect on the distribution of dendritic mitochondria in comparison to WT (arrowheads, Fig. A7A). In contrast, dMiro OE depleted dendrites of mitochondria (Fig. A7A). The effects on
Figure A6. Effects of dMiroT25N and dMiroT460N on dynein-driven motility during retrograde axonal transport of mitochondria. (A-H), mitoGFP was co-expressed with myc-dMiro (control, dMiro, -/-), -T25N or -T460N in MNs of dmiro null mutants (Null (-/-)) using an Ok6-Gal4 driver. Confocal time-lapse images of mitoGFP-tagged mitochondria in motor axons were acquired at a rate of 1 sec\(^{-1}\) as described previously. Asterisks indicate significant differences among indicated genotypes (Kruskal-Wallis and Dunn’s post test). (A), net-velocity of retrograde axonal mitochondrial transport (p<0.05, N>36). (B-C), fraction of time mitochondria spent on MT minus end-directed trips (B) and short stops (C), during retrograde transport (p<0.05, N>20). (D), average duration of stops of mitochondria during retrograde transport (p<0.01, N>18). (E-F), distance of MT minus end-directed cRuns (E) and trips (F) during retrograde transport (p<0.05, N>36). (G), number of MT minus end-directed cRuns during retrograde transport (p<0.05, N>40). (H), Fraction of MT minus end-directed trips that exhibited cRuns (p>0.05, N>29).
the mitochondrial distribution induced by dMiroT25N OE were not consistent with those expected by a dominant-negative mutation.

To better test whether dMiroT25N resembles a dominant-negative mutation, we examined to what degree its phenotypic effects on anterograde mitochondrial transport in MN axons are sensitive to the presence of endogenous dMiro. Pairwise comparison showed that the presence of wild type protein strongly attenuated the severe impairments induced by dMiroT25N expression in dmiro nulls (p<0.05; Fig. A7B-I). This strong attenuation of dMiroT25N phenotypes is remarkable since dMiroT25N expression levels were much higher than those of...
endogenous dMiro (Fig. A1D-E). Nevertheless, dMiroT25N OE still caused some dominant effect. In comparison to wild type and dMiro control, dMiroT25N OE reduced the net-velocity of transport and distance of kinesin-driven cRuns while it increased the time mitochondria spent on kinesin-driven trips (p<0.01, Fig. A7C-E). Importantly, the latter was qualitatively the opposite effect of its expression in dmiro nulls. Hence, the phenotypic effects of dMiro OE overall are inconsistent with those of a dominant-negative mutation.

Notably, dMiroT25N OE and dMiro OE had essentially the same effect on number of kinesin-driven cRuns, the percentage of kinesin-driven trips containing cRuns, and the number of cRuns per trip (p<0.05 Fig. A7G-I), suggesting that these effects are exclusively caused by dMiro OE and do not require the activity of the N-terminal GTPase domain.

dMiro’s N-terminal GTPase domain is required for the dynein-dependent distribution of dendritic mitochondria in sensory neurons.

The differential effects of dMiroT25N expression in dmiro nulls on kinesin- and dynein-driven axonal transport of mitochondria (Figs. A5-A6) could have been caused by an exclusive defect in kinesin motility, since dynein-driven retrograde transport of mitochondria in fly MN axons depends on normal kinesin activity (Pilling et al., 2006). However, it also was possible that dMiroT25N may prevent both kinesin- and dynein-driven transport because dMiroT25N failed to properly distribute mitochondria into MN dendrites (Fig. A2A). To better resolve the significance of the N-terminal GTPase domain for dynein-driven transport, we turned to multi-dendritic sensory neurons (SNs).

Mitochondria of multi-dendritic SNs (class I, vpda; Grueber et al., 2002) were visualized by transgenic expression of mitoGFP using the 21-7 Gal4 driver (Song et al., 2007). The examined SNs are located in the larval body wall and feature a single axon and a major primary
Figure A8. Effects of dMiroT25N on the distribution of mitochondria in dendrites of sensory neurons. (A-H), mitoGFP was transgenically co-expressed with normal myc-dMiro (control), myc-dMiroT25N, or dmiro promoter-driven genomic transgene expressing only dMiro-PC in vpdfa SNs of dmiro null mutants (Null (-/-)) using the indicated 21-7 and C380 Gal4 drivers. Mitochondria in larval SNs were visualized by confocal imaging of immunostained larval body wall preparations using anti-GFP and anti-HRP antibodies. Asterisks indicate significant
dendrite (arrowheads) that emerges from the soma (arrow) and gives rise to a number of uniquely organized secondary branches that run almost parallel to each other (Fig. A8A). In contrast to mammalian dendrites, larval SN dendrites exhibit a MT minus-end-out organization and require dynein for anterograde transport of mitochondria (Grueber et al., 2002; Rolls et al., 2007; Satoh et al., 2008; Stone et al., 2008; Rolls, 2011).

In wild type SNs, mitoGFP-tagged mitochondria were found throughout primary and secondary dendrites in irregularly spaced intervals (Fig. A8A). However, SNs of dmio null mutants exhibited a severe depletion of dendritic as well as axonal mitochondria that was accompanied by an accumulation of somatic mitochondria (Fig. A8A-B). The few dendritic mitochondria were mainly constrained to proximal regions of the primary dendrite, and rarely present in secondary or tertiary dendrites (Fig. A8A, C-E). The loss of dendritic mitochondria in dmio null mutant SNs was associated with a structural impairment of the dendritic tree, reducing the average length of secondary but not primary dendrites (Fig. A8F-G), which together reduced the overall size of the dendritic tree (Fig. A8H). Notably, a similar impairment of the dendritic tree has been observed after knockdown of TRAK2 in cultured hippocampal neurons (van Spronsen et al., 2013).

Expression of normal dMiro in dmio null mutant SNs from a transgene containing a dmio promoter restored the distribution of mitochondria in both axons (not shown) and dendrites, and even caused a significant increase in the amount of dendritic mitochondria (Fig.
Hence, this suggests that dMiro is required for both the dynein-driven dendritic and the kinesin-driven axonal distribution of mitochondria.

Notably, 21-7 Gal4-driven expression of normal dMiro in dmiro null mutant SNs restored the distribution of mitochondria in axons but not in dendrites (Fig. A8A-B). This phenomenon mirrored the effects of Ok6 Gal4-driven expression of dMiro in dmiro null mutant MNs (Fig. A2A). In both cases, the depletion of dendritic mitochondria was caused by a dominant overexpression effect since both 21-7-Gal4 and Ok6-Gal4-driven overexpression of dMiro induced a severe depletion of mitochondria in dendrites but not axons of otherwise wild type SNs and MNs (Figs. A9A-C, A7A, respectively). To minimize the effects of dMiro OE on
dendritic mitochondria, we tested a number of Gal4 drivers including 477, 2-21, 5-40 and C380 (Grueber et al., 2003; Song et al., 2007; Sanyal, 2009). Of these, only C380 Gal4-driven expression attenuated the dMiro OE effect and partially restored the dendritic distribution of mitochondria in comparison to dmiro nulls (p<0.05) to 45.7% of wild type levels (p<0.001, Fig. A8A, C-E).

To test the requirement of dMiro’s N-terminal GTPase domain for distributing mitochondria into dendrites, we expressed dMiroT25N in dmiro null mutant SNs using both the 21-7 and C380 Gal4 driver. However, C380-driven dMiroT25N expression had essentially no effect on the reduced amount of dendritic mitochondria in comparison to dmiro null mutant SNs (p>0.7), and was significantly different from the partial rescue induced by normal dMiro expression (p<0.001, Fig. A8A, C-E). 21-7 driven expression causes a similar depletion of dendritic mitochondria (not shown). Importantly, both 21-7 and C380-driven (not shown) expression of dMiroT25N also failed to restore the distribution of mitochondria into axons of the examined SNs (Fig. 8B), which mirrored the effects of dMiroT25N expression in dmiro null mutant MNs (Fig. A2B-G). Together these data suggest that dMiro’s N-terminal GTPase domain is required for both the kinesin- and the dynein-driven distribution of mitochondria into axons and dendrites, respectively.

Notably, dMiroT25N OE disrupted the dominant effect of normal dMiro OE on dendritic mitochondria in both MNs and SNs (Figs. A9A, C, A7A, respectively). In SNs, dMiroT25N OE still caused a slight reduction to 73% of control levels (Fig. A9A, C). The remaining effect was likely still a consequence of increased dMiro-mediated kinesin linkage since T25N did not disrupt the increase in kinesin-driven trip motility induced by dMiro OE (Fig. A7F-I). Nevertheless, the effect of dMiroT25N OE was significantly different to the almost complete depletion of dendritic mitochondria induced by dMiro OE (p<0.001, Fig. A9C). dMiroT25N OE
had no effect on the length of the dendritic tree (p=0.7), which was significantly reduced by normal dMiro OE (p<0.01, Fig. A9D).

dMiro’s C-terminal GTPase domain modulates retrograde mitochondrial transport in axons.

Expression of the C-terminal GTPase mutation dMiroT460N in dmiro nulls restored a normal density of motile and stationary mitochondria in proximal axons of MNs (p>0.9, Fig. A4A-C) and facilitated a normal flux of anterograde mitochondrial transport in comparison to normal dMiro (p>0.2, Fig. A4D).

Consistent with a normal axonal mitochondrial distribution, dMiroT460N had no effect on the net-velocity, the preferential use of kinesin motors and the distance of kinesin-driven cRuns and trips in comparison to normal dMiro (p>0.3, Fig. A5A-C, F-G). However, dMiroT460N slightly but significantly reduced the distance of kinesin-driven iRuns (data not shown) while it increased the duration of stops and the time mitochondria spent motionless (p<0.05, Fig. A5D-E). Notably, dMiroT460N increased the number of kinesin-mediated cRuns and the percentage of kinesin trips with cRuns (p<0.01, Fig. A5H-I). However, the resulting increase in the distance of kinesin-mediated trips was not significantly different to control (p>0.05, Fig. A5G).

Taken together, the almost perfect rescue of dmiro null phenotypes by dMiroT460N expression suggest that dMiro’s C-terminal GTPase domain may be largely dispensable for anterograde axonal transport of mitochondria. Nevertheless, the dMiroT460N induced increase of the number of kinesin-driven cRuns and the percentage of trips with cRuns indicated a potential modulatory role (Fig. A5H-I). However, this may not be the case since dMiroT460N OE affected anterograde transport in a paradoxical way. dMiroT460N OE reduced the net velocity of anterograde transport and the distance of kinesin-driven cRuns in comparison to dMiro OE, wild
type control and dMiroT460N expression in nulls (p<0.01; Fig. A7C). In addition, dMiroT460N OE reduced the number of cRuns in comparison to dMiro OE (p<0.01; Fig. A7G) to wild type levels while expression in *dmiro* nulls caused a significant increase in comparison to dMiro OE (p<0.01; Fig. A5H). These paradoxical phenotypic effects of dMiroT460N are inconsistent with a role of the C-terminal GTPase domain modulating kinesin-driven transport. Instead, they may be a consequence of a transport-unrelated role of the domain.

In contrast to anterograde transport, dMiroT460N expression in *dmiro* nulls significantly impaired retrograde mitochondrial transport. In comparison to dMiro control, it reduced the net velocity, the time spent on dynein-mediated trips and the distance of dynein-mediated trips (p<0.05, Fig. A6A-B, F). dMiroT460N also caused an increase in the time mitochondria spent on stops (Fig. 6C). All of these effects were significantly less severe than those of *dmiro* nulls (p<0.05, Fig. A6A-F). dMiroT460N had no significant effect on the duration of stops, the distance of dynein-driven cRuns, the average number of cRuns or the fraction of dynein-mediated trips containing cRuns (p>0.05; Fig. A6D-E, G-H). Together, these data suggest that dMiro’s C-terminal GTPase domain modulates retrograde transport, possibly by modulating dynein motility or other aspects of mitochondrial biology.

Expression of dMiroA20V and dMiroK455V causes neomorphic gain of function effects.

The presumed constitutively active mutations dMiroA20V and dMiroK455V did not facilitate normal mitochondrial transport in axons of MNs, even though they facilitated a normal distribution of mitochondria in axons and NMJs (Fig. A2). In comparison to normal dMiro, expression of dMiroA20V and -K455V in *dmiro* nulls significantly reduced the density of motile (p<0.001) but not stationary mitochondria in proximal axons (p>0.05, Fig. A4B-C). Both mutations also severely reduced the flux of antero- and retrograde axonal transport (p<0.001,
Figure A10. Effects of dMiroA20V and dMiroK455V on kinesin-driven motility during anterograde axonal transport of mitochondria. (A-F), myc-dMiro (control), -A20V or -K455V were co-expressed with mitoGFP in MNs of dmiro null mutants (dMiro-xx, -/-) or overexpressed (dMiro-xx OE) in MNs of wild type control (w1118) using an Ok6-Gal4 driver. Confocal time-lapse images of mitoGFP-tagged mitochondria in motor axons were acquired at a rate of 1 sec⁻¹. Asterisks indicate significant differences for the pairwise comparison between effects induced by expression in a null and wild type background (Mann-Whitney), and differences from WT and dMiro OE among all indicated genotypes (Kruskal-Wallis and Dunn’s post test). (A), mitochondrial flux of anterograde transport in motor axons of proximal larval nerves (p<0.001, N>6). (B), net-velocity of anterograde mitochondrial transport in axons (p<0.05, n>29, N>6). (C), fraction of time mitochondria spent on MT plus end-directed trips during anterograde transport (p<0.05, n>29, N>6). (D-E), distance of MT plus end-directed runs (D) and trips (E) during anterograde mitochondrial transport (p<0.001, n>29, N>6). (F), number of MT plus end-directed cRuns during anterograde transport (p<0.01, n>29, N>6).
Furthermore, dMiroA20V and -K455V reduced the net velocity of anterograde transport \((p<0.001)\), the time mitochondria spent on kinesin-driven trips \((p<0.05)\) and the distance of kinesin-driven runs and trips to a similar degree \((p<0.001, \text{Fig. A10B-E})\). Retrograde axonal transport was equally impaired as both dMiroA20V and dMiroK455V reduced the net velocity \((p<0.001)\) and the distance of dynein-driven runs \((p<0.001)\) and trips \((p<0.05, \text{not shown})\).

Since the effects of dMiroA20V and -K455V on axonal mitochondrial transport were difficult to reconcile with those of the corresponding loss of function mutations dMiroT25N and -T460N, we examined their genetic nature by comparing their phenotypic effects in the absence and presence of endogenous dMiro. Pairwise comparison showed that expression of dMiroA20V and -K455V in wild type and dmiro null mutants had essentially the same effects on anterograde \((p>0.05; \text{Fig. A10A-F})\) and retrograde transport of mitochondria (data not shown). Hence, the phenotypic effects of dMiroA20V and -K455V are inconsistent with those of a constitutively active or hypermorphic mutation, whose phenotypes are typically enhanced by increased levels of endogenous wild type protein. Instead, both mutations exhibited features of a neomorphic gain of function mutation (Muller, 1932). Therefore, we refrain from the inherently difficult interpretation of these neomorphic phenotypes since they are often due to the acquisition of a new function that is unrelated to the normal function of the protein (Muller, 1932; Wilkie, 1994).

**Mutations in dMiro’s C-terminal GTPase domain do not modulate the phenotypic effects of mutations in the N-terminal GTPase domain.**

Our genetic analysis of dMiroT460N suggests that dMiro’s C-terminal GTPase domain is not critical for anterograde mitochondrial transport. Nevertheless, the domain could still cooperate
with the N-terminal GTPase domain. To test this, we generated the double mutant transgenes dMiroT25N-T460N, dMiroT25N-K455V, dMiroA20V-T460N and dMiroA20V-K455V. The double mutant proteins were localized properly to mitochondria, and their expression levels were slightly higher than those of the respective single mutant dMiro proteins (1.5-2-fold of dMiro control, not shown).
Expression of dMiroT25N-T460N and dMiroT25N-K455V in MNs of dmiro null mutants caused a pronounced accumulation of mitochondria in the soma and a severe depletion in dendrites and axons (Fig. A11A). Proximal axons contained only very few mitochondria while NMJs did not contain more than 1-3 sporadic mitochondria (Fig A11B). All of these phenotypic effects were indistinguishable to those of dMiroT25N (p>0.05; N>5). Hence, the mutations T460N and K455V are not able to modulate the pronounced inhibition of mitochondrial transport that is caused by the T25N-induced inactivity of the N-terminal GTPase domain. These finding do not provide evidence for a cooperative action of dMiro’s N- and C-terminal GTPase in control of mitochondrial transport.

Expression of dMiroA20V-T460N and dMiroA20V-K455V in MNs of dmiro null mutants facilitated the distribution of mitochondria into axons and NMJs (Fig. A11C). In comparison to dMiroA20V, both mutations increased the size of synaptic boutons (p<0.001, Fig A11E), and the area occupied by mitochondria, although the latter was only significant for dMiroA20V-T460N (p<0.01, Fig A11D). Despite the accumulation of mitochondria at axon terminals, both dMiroA20V-T460N and dMiroA20V-K455V impaired mitochondrial transport in a manner that was indistinguishable from dMiroA20V (p>0.05, Fig. A11F-I), which is consistent with its proposed neomorphic nature.

Notably, dMiroA20V-T460N expression induced a significant sensitivity of synaptic mitochondria to standard formaldehyde fixation, as it was used throughout this study (Fig. A11C). This problem could be fully eliminated by using Bouin’s fixative (Fig. A11C), which visualized a similar amount of synaptic mitochondria as live imaging (not shown). The cause of this phenomenon is unclear but could be caused by an alteration in the lipid composition of A20V-T460N-mutant mitochondrial membranes, assuming that the domains are required for
lipid exchange between the ER and mitochondria, as it has been demonstrated for yeast Miro (Kornmann et al., 2011).

A.5 Discussion

Previous studies demonstrated a critical role of dMiro for the kinesin-mediated distribution of mitochondria into axons (Guo et al., 2005a; Russo et al., 2009b). Here we extend these studies and show that dMiro is also required for the dynein-dependent distribution of mitochondria into dendrites of SNs. In addition, we used the mutations dMiroT25N and dMiroT460N to determine the biological significance of dMiro’s N- and C-terminal GTPase domains. Our analysis suggests a critical neuronal role of dMiro’s N-terminal GTPase domain for survival, for normal distribution of mitochondria into axons and dendrites, and for maintaining a normal mitochondrial size. Overall, our analysis indicates that dMiro’s N-terminal GTPase domain facilitates the transport of mitochondria by promoting their transition from a stationary to a motile state irrespective of the employed motor. In contrast, our analysis of dMiroT460N suggests that dMiro’s C-terminal GTPase domain is not essential for viability and the distribution of mitochondria into neuronal processes, although a subtle modulatory role for retrograde mitochondrial transport is indicated. Finally, we show that amino acid substitutions analogous to the constitutively active Ras-G12V mutation in dMiro’s N- and C-terminal GTPase domains cause neomorphic phenotypic effects that are likely unrelated to the normal function of both GTPase domains.

Genetic nature of the mutations in dMiro’s GTPase domains.

The dominant-negative Ras-S17N and constitutively active Ras-G12V mutations served as templates for altering the activities of Miro’s two GTPase domains in yeast, flies and mammals (Fransson et al., 2003; Fransson et al., 2006; MacAskill et al., 2009a; Kornmann et al., 2011;
Koshiba et al., 2011; Murley et al., 2013). Studies of mammalian Miro overexpressing these GTPase mutant proteins in various cell culture systems reported phenotypic effects that were conceptually difficult to reconcile (Fransson et al., 2003; Fransson et al., 2006; MacAskill et al., 2009a). Therefore, we genetically verified the potential dominant nature of the analogous fly mutations.

Expression of the RasS17N analog dMiroT25N in dmiro null mutants was lethal and severely impaired the distribution and transport of mitochondria in neurons. Remarkably, these effects were strongly suppressed by the presence of endogenous dMiro, suggesting that T25N is essentially a recessive loss of function mutation. The recessive nature of dMiroT25N is not a species-specific exception, since the analogous mutation in yeast Miro also had no dominant effects (Koshiba et al., 2011). A potentially recessive nature of the analogous mammalian Miro18N mutation may explain its wild type-like overexpression effects on the mitochondrial distribution in cultured hippocampal neurons (MacAskill et al., 2009a). A similar classification could not be done for dMiroT460N.

Expression of the Ras-G12V analog dMiroA20V in dmiro nulls facilitated the distribution of mitochondria into neuronal processes like normal dMiro. However, unlike normal dMiro, dMiroA20V expression in both dmiro nulls and wild type animals impaired viability and mitochondrial transport to a similar degree, which is a characteristic feature of a neomorphic mutation, and inconsistent with a constitutively active mutation (Muller, 1932; Wilkie, 1994). Neomorphic effects are typically not related to normal protein function since they are often due to the acquisition of a toxic protein feature or an unnatural proteininteraction (Wilkie, 1994). However, a constitutively active GTPase domain could cause neomorphic effects too. Since GTPase signaling is not meant to operate continuously, a constitutive activity could induce
negative-feedback mechanisms counteracting the effects of its chronic activation (Cantrell, 2002).

Remarkably, dMiroK455V had neomorphic effects on mitochondrial transport and structure that were essentially indistinguishable to those of dMiroA20V, and showed no additive effects when combined in a double mutant protein. The similar effects of dMiroA20V and -K455V contrasted the fundamentally different effects of the corresponding loss of function mutations, dMiroT25N and -T460N, which either prevented or permitted transport, respectively. Hence, the significantly different functional requirement of GTPase domains makes a potential constitutively active nature of dMiroA20V and –K455V unlikely.

How could dMiroA20V and -K455V induce similar dominant phenotypes, if only the N-terminal GTPase domain is required for anterograde transport? The neomorphic character of both mutations might provide an explanation. Since both GTPase domains constitute Ras-like domains (Reis et al., 2009; Klosowiak et al., 2013), one can expect that the analogous substitutions A20V and K455V may cause a similar molecular change in each domain. Accordingly, it seems possible that the similar neomorphic effects are caused by the acquisition of either a similar toxic protein feature or a similar unnatural protein interaction, and not by a constitutively active GTPase domain.

dMiro’s N-terminal GTPase domain mediates the mobilization of stationary mitochondria.

Miro is one of at least two factors that ascertain mitochondrial motor linkage through its association with the Milton/TRAK motor adaptor complex (Fransson et al., 2006; Glater et al., 2006; MacAskill et al., 2009a; MacAskill et al., 2009b; Wang and Schwarz, 2009; Koutsopoulos et al., 2010; van Spronsen et al., 2013). At least in mammals, polarized mitochondrial transport into either axons or dendrites is achieved by differential interactions of TRAK1 and 2 with
kinesin and dynein motors (van Spronsen et al., 2013). The activity of the linked motors is in part controlled by Ca$^{2+}$ activation of Miro’s EF-hands arresting transport (MacAskill et al., 2009b; Wang and Schwarz, 2009). However, the significance of Miro’s GTPase domains for mitochondrial transport remained unclear.

Previous mammalian studies indicated that Miro’s GTPase domains do not control the association of Miro with TRAK proteins. Mutations in the N- (N18) and C-terminal GTPase domain (N432) of Miro 1 and 2 had no effect on the ability to co-precipitate TRAK1 or 2 (Fransson et al., 2006). In vivo, mycMiro1-N18 also failed to affect the mitochondrial recruitment of cytosolic YFP-TRAK2 (MacAskill et al., 2009a). Our analysis of the MiroN18-analogous fly mutation dMiroT25N revealed that a normal activity of Miro’s N-terminal GTPase domain is critical for survival, mitochondrial size and transport, likely by controlling the transition of mitochondria from a stationary to a motile state.

dMiroT25N expression in the absence of endogenous dMiro caused an accumulation of mitochondria in the soma of MNs and SNs, while axons and dendrites were severely depleted of mitochondria. The few motile mitochondria found in MN axons exhibited defects in both kinesin and dynein motility. Hence, it is unlikely that dMiro’s N-terminal GTPase domain resembles a binary switch that controls exclusively kinesin motility or switches mitochondrial motility from a kinesin- to a dynein-driven state. Instead, these findings argue that the N-terminal domain controls a fundamentally different mechanism that mediates the transition from a stationary to a motile state irrespective of the identity of the subsequently employed MT motor. Accordingly, Miro may mediate mitochondrial transport by providing a membrane anchor linking motors to mitochondria, by controlling the activity of linked motors through its EF hand domains, and by controlling the transition from a stationary to a motile state through a yet unknown mechanism.
How might dMiro’s N-terminal GTPase domain mobilize mitochondria?

The transition of stationary mitochondria to a motile state could be principally achieved by controlling either the recruitment of motors to immobile mitochondria, the activity of the linked motors, or the detachment of mitochondria from a stationary site. The first possibility is unlikely since loss of Miro’s N-terminal GTPase activity did not inhibit its association with TRAK motor complexes (Fransson et al., 2006; MacAskill et al., 2009a). Instead, the N-terminal domain could control the activity of the linked motors in a manner similar to Miro’s EF-hand domains, by either disengaging motors from MTs, or by triggering their release from Milton/TRAK adaptors. This possibility is supported by the severe motility defects of the few axonal dMiroT25N-mutant mitochondria escaping the stationary state. Alternatively, the N-terminal domain could detach mitochondria from a stationary site, which is reminiscent of Miro’s role in yeast, where it acts as a negative regulator of a physical ER-mitochondria link (Kornmann et al., 2011; Murley et al., 2013).

The roles of the GTPase domains in yeast Miro are well defined. Yeast mitochondria are physically tethered to the ER by the multi-protein ERMES complex, which is required for ER-associated phospholipid exchange, mitochondrial division, and the association with actin. Both the N- and to a lesser degree the C-terminal domain are required for the dissociation of mitochondrial ER contacts and the maintenance of mitochondrial morphology (Boldogh et al., 2003; Frederick et al., 2004; Kornmann et al., 2009; Kornmann et al., 2011; Toulmay and Prinz, 2012; Voss et al., 2012; Murley et al., 2013).

Metazoan Miro may also dissociate ER–mitochondria contacts, since mammalian Miro1 expressed in yeast is localized to ER–mitochondria interfaces (Kornmann et al., 2011). Accordingly, the accumulation of mitochondria in neuronal somata of dMiroT25N and dmiro null mutants could be a consequence of abnormal ER–mitochondria interactions downstream of ER-
associated mitochondrial division and upstream of mitochondrial dispersion by motor proteins. Like in yeast mutants, dMiroT25N- and dmiro null-mutant mitochondria were severely fragmented, which is consistent with a function downstream of mitochondrial division. However, further studies will be needed to elucidate the true nature of the immobilized state induced by the loss of dMiro’s N-terminal GTPase activity.
APPENDIX B: STATEMENT OF PERMISSIONS

Per the Permissions Policy of the Journal of Neuroscience (available at http://www.jneurosci.org/site/misc/Permissions_Policy.pdf), authors are automatically granted permission to use their articles in a thesis and/or dissertation. The article attached in Appendix A is used under the aegis of this policy.
REFERENCES


Cherfils J, Zeghouf M (2013) Regulation of small GTPases by GEFs, GAPs, and GDIs. Physiol Rev 93:269-309.


mitochondrial membrane potential depolarization and stimulates Parkin E3 ligase activity by phosphorylating Serine 65. Open Biol 2:120080.


Narendra D, Kane LA, Hauser DN, Fearnley IM, Youle RJ (2010a) p62/SQSTM1 is required for Parkin-induced mitochondrial clustering but not mitophagy; VDAC1 is dispensable for both. Autophagy 6:1090-1106.


Valente EM et al. (2002b) PARK6-linked parkinsonism occurs in several European families. Ann Neurol 51:14-18.


Yoneda M, Miyatake T, Attardi G (1994) Complementation of mutant and wild-type human mitochondrial DNAs coexisting since the mutation event and lack of complementation of DNAs introduced separately into a cell within distinct organelles. Mol Cell Biol 14:2699-2712.


