Functional analysis of Miro GTPase domains in the mitochondrial motor adaptor complex

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Functional analysis of Miro GTPase domains in the mitochondrial motor adaptor complex

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Functional analysis of Miro GTPase domains in the mitochondrial motor adaptor complex

A dissertation presented

by

Kayla Janae Davis

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Dissertation Advisor: Thomas Schwarz **Kayla Janae Davis**

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Abstract

Mitochondria are actively transported in all somatic cells where their motility is essential for proper cellular function. Mitochondrial transport relies on microtubules and a highly regulated motor/adaptor complex that consists of Miro1, a mitochondrial outer membrane protein; TRAK1/2, the adaptor proteins; and kinesin-1, and dynein, the molecular motors. Miro1 and its family member Miro2 are Rho like GTPases that contains two GTPase domains (N-GTPase and C- GTPase) that are separated by EF hands. Although the GTPase domains of Miro are a prominent feature, their functional significance is controversial. During the course of my thesis, I have uncovered a novel mechanism by which the Miro1 N-GTPase regulates assembly of the motor/adaptor complex. Although Miro2 has long been assumed to play a similar role to Miro1 in mitochondrial motility, I have found that Miro2 does not interact with components of the motor/adaptor complex in the same way as Miro1. To study the function of the Miro GTPase domains without confounding influences from the presence of endogenous mitochondrial Miro, I've misdirected Miro1 and Miro2 to peroxisomes with the transmembrane domain of PEX3. When PEX3-Miro1 is co-expressed with components of the motor/adaptor complex, TRAK1/2 and the motors kinesin-1 and dynein (P135), these proteins are also re-directed to peroxisomes. Using the peroxisome-directed Miro1, we find that the Miro1 N- GTPase regulates the ability of Miro1 to interact with the rest of the motor adaptor complex: neither TRAK1/2 nor kinesin or

P135 colocalize on peroxisomes with the GDP-locked (T18N) mutation. In addition to a loss of interaction with the motor/adaptor complex, the distribution of peroxisomes in COS-7 cells and hippocampal neurons are correspondingly affected. The complex assembles correctly with the GTP-locked N-GTPase (P13V) mutation. In contrast, neither the GTP-locked nor GDP-locked C-GTPase mutations substantially alter Miro1 recruitment of TRAK1/2 or kinesin to peroxisomes. We find that PEX3-Miro2 is unable to recruit the components of the motor/adaptor complex to peroxisomes regardless of the state of its N-terminal or C-terminal GTPase. Thus, N-GTPase of Miro1 is critical for regulating Miro1's interaction with the other components of the motor/adaptor complex and thereby for regulating mitochondrial motility.

Contents

Dedication

To my parents and grandparents, who have supported me endlessly and without question in my educational and professional endeavors, and my brother, who was brave enough to do it first and showed me it could be done.

To my friend John Hatch, who did not get the chance to write his own thesis but whose encouragement and support influenced this one.

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List of figures and tables

Chapter 1.
Introduction

The role of mitochondria in cellular homeostasis

Mitochondria are critical for eukaryotic cell survival; they regulate cellular metabolism and produce energy by supplying the ATP necessary for proper cellular function. Maintaining energy homeostasis requires efficient regulation of mitochondrial trafficking and anchoring so that mitochondria can be easily and quickly recruited and then redistributed as metabolic demands change. In neurons, mitochondria are not only tasked with supplying billions of molecules of ATP per second but also play an essential role in mediating calcium homeostasis during neurotransmission and short-term plasticity (Emptage et al., 2001; MacAskill et al., 2009; Mattson et al., 2008; Mochinda et al., 2008; Schwarz, 2013; Wang et al., 2009). Many studies have linked disrupted mitochondrial trafficking in neurons with axonal degeneration and dysfunctional synaptic transmission and, in particular, to the pathologies of Alzheimer's disease, amyotrophic lateral sclerosis (ALS) (Morotz et al., 2012; Zhang et al., 2015), and Parkinson's disease (Hardy, 2010; Liu et al., 2012; Nguyen et al., 2014; Wang et al., 2011). These studies illustrate the critical nature of maintaining the proper regulation of mitochondrial trafficking for cellular health.

Within cells, mitochondrial motility is highly regulated. In neurons, mitochondria are produced in the soma and then migrate outwards into axons and dendrites to energetically active regions (Misgeld and Schwarz, 2017). Mitochondria can move quickly, particularly through neuronal axons, where they may travel up to a meter in distance from the soma to reach synaptic terminals. They can travel at speeds up to 1 μm/ second (Misgeld and Schwarz, 2017). In non-neuronal cells, mitochondria are in highly organized reticular structures and move to areas requiring energy, like the leading edge of migrating cells (Cunniff et al., 2016). Mitochondria

can quickly transition their motile state to paused, where they stop for only a short time and then continue, or stationary and immobile where they will stay in place for extended periods (Gutnick et al., 2019; Hollenbeck, 1996; Morris and Hollenbeck, 1995; Ligon et al., 2000; Hollenbeck and Saxton, 2005). A motor-adaptor complex mediates the transport of mitochondria. Although the essential components of the mitochondrial motor-adaptor complex have been characterized, there are still many questions about how the individual proteins in the motor-adaptor complex work together to regulate mitochondrial motility. These questions extend to how components of the motor-adaptor complex might interact with other organelles. Because mitochondrial motility is critical for cell health and proper cellular function, it is crucial to continue studying the mechanisms regulating mitochondrial motility to best understand how the regulation of mitochondrial trafficking and the mitochondrial motor-adaptor complex relates to disease.

The mitochondrial motor-adaptor complex

The transport of mitochondria relies on a motor-adaptor complex and the microtubule cytoskeleton. Mitochondria travel along microtubule tracks, polymers of α- and β-tubulin that form into long cylindrical filaments. Microtubules are highly dynamic in length, growing through polymerization and shrinking through depolymerization of tubulin. They are highly polar and extend outwards from the plus (+) end, while their minus (-) end remains stable. Microtubules are typically about 25nm in width and can grow to be long filamentous tracks, spanning 100's of um's in length in specific cell types (Schwarz, 2013). These microtubule tracks serve as roadways for motor proteins that carry cargos, like organelles, proteins, viruses, DNA, and RNA throughout cells (Farina et al., 2013; Fukuda et al., 2020; Goodson and Johnson, 2018; Kanai et al., 2004; Shimert et al., 2019). Mitochondria are transported towards the plus (+) ends

of microtubules in what is referred to as anterograde movement by the molecular motor, kinesin-1 (Glater et al., 2006; Hirokawa et al., 1991; Hurd and Saxton, 1996; Pillling et al., 2006 Tanaka et al., 1998). The kinesin-1 motor takes steps forward on microtubules by hydrolyzing ATP in its N-terminal motor domains. Members of the kinesin-1 family include KIF5A, KIF5B, and KIF5C, with KIF5B expressed ubiquitously across cell types and KIF5A and KIF5C expressed only in neurons (Brady, S. T, 1985; Vale et al., 1985; Lin and Sheng, 2015).

In contrast, mitochondria are transported towards the minus (-) end of microtubules in what is referred to as retrograde movement by the dynein transport machine, made up of the dynein complex and the dynactin complex (Drerup et al., 2017; Pilling et al., 2006; Reck-Peterson et al., 2018). These motors are recruited to the outer mitochondrial membrane by the adaptor protein TRAK1/2 (also called Milton or TRAK1/OIP106, TRAK2/GRIF1), which links molecular motors to Miro, a mitochondrial outer membrane protein and Rho-like GTPase (figure 1.1) (Glater et al., 2006; Stowers et al., 2002; van Spronsen et al., 2013). These mitochondrial motor-adaptor complex proteins are conserved in *Drosophila* and mammals (Hirokawa et al., 1991; Pilling et al., 2006; Smith et al., 2006; Stowers et al., 2002).

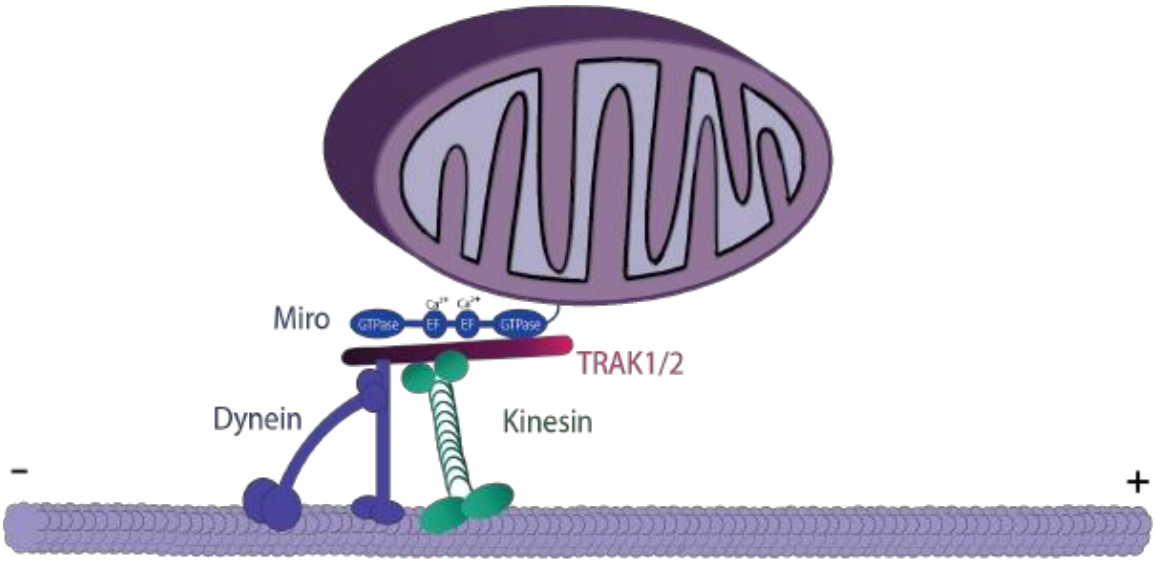


Figure 1.1 Cartoon of the mitochondrial motor-adaptor complex
The mitochondrial motor-adaptor complex comprises Miro1, the mitochondrial outer membrane protein, TRAK1/2 the adaptor protein, and the molecular motors kinesin and dynein. Miro1 has a C-terminal transmembrane domain anchored into the mitochondrial outer membrane, two GTPase domains, referred to as the N-terminal GTPase domain and the C-terminal GTPase domain, and two pairs of EF-hands, which sit in-between the GTPase domains. Miro1 interacts with the protein TRAK1/2. TRAK1/2 has two coiled-coil (CC) domains where the molecular motors kinesin and dynein interact. Kinesin interacts with TRAK1 at its N-terminal CC domain and carries mitochondria towards microtubule plus-ends. Dynein also interacts with TRAK1 at its N-terminal CC domain and moves mitochondria towards microtubule minus-ends.

TRAK1/2: the mitochondrial motor-adaptor

Together, the four-protein mitochondrial motor-adaptor complex is critical for efficient mitochondrial motility. Without an adaptor, the kinesin-1 motor is typically auto-inhibited and incapable of moving along microtubules independently (Henrichs et al., 2020). It has been shown that kinesin-1's interaction with TRAK1 dramatically improves its performance for making long-distance runs and increases the motor protein's efficiency when encountering obstacles along microtubules (Henrichs et al., 2020). These findings give evidence for why understanding the cohesive interaction of the entire motor-adaptor complex is essential for understanding mitochondrial motility. TRAK1 has a homologue, TRAK2, with which it shares 44% sequence consensus. Both proteins contain a coiled-coil domain and have been shown to be an adaptor for

kinesin (Glater et al., 2006; Randall et al., 2013; Stowers et al., 2002). TRAK1 and TRAK2 are both ubiquitously expressed throughout tissues. In developing and adult brain, TRAK2 is more dominantly expressed in cerebellum, cortex, and midbrain. Using western blot analysis, TRAK1 was found to be more highly expressed than TRAK2 in several organs including the heart, liver, lungs, and spleen. In these same western blot expression experiments, TRAK1 was found to be more highly expressed in the nervous system than TRAK2, including cerebral gray and cervical spinal white matter (van Spronsen et al., 2013). There are three TRAK1 splicing variants, which vary in length (figure 1.2). The longest TRAK1 splicing variant (var-1) is found to be dominantly expressed in neonatal tissue. During development the TRAK1 var-1 expression decreases and TRAK1 splicing variant 2 (var-2) is more highly expressed in adult tissue. (Blue et al., 2019) (figure 1.2) For all experiments in this thesis, we have used TRAK1 splicing variant 1. TRAK1 and TRAK2 have been shown to interact with KIF5A, KIF5B, and KIF5C in different tissues (Brickley et al., 2005; MacAskill et al., 2008). Disruption of Miro1 and TRAK1 interaction leads to a decreased mitochondrial trafficking phenotype (Glater et al., 2006; MacAskill et al., 2008) In neurons, TRAK1 has primarily been found in axons and is believed to be the dominant adaptor for interaction with kinesin. In contrast, TRAK2 has been found to primarily interact with dynein and be involved in dendritic localization of mitochondria (van Spronsen et al., 2012). This difference in binding to the kinesin motor has been described as a result of a TRAK2 conformational change where the protein folds in half, blocking the TRAK2 and kinesin binding domain, thus regulating directional motility of mitochondria (van Spronsen et al., 2012). Recent studies have found that an increase in caspase-cleaved tau decreases the expression of TRAK2 but not TRAK1, Miro, kinesin, or dynein, and that this increase in caspase cleaved tau leads to a re-localization of mitochondria to the soma of neurons, suggesting that the

TRAK1/2 adaptor proteins are differentially regulated (Quintanilla et al., 2020). Together, these results suggest a functional difference in TRAK1 and TRAK2 and their regulation of mitochondrial motility, which has yet to be fully elucidated. In chapter 2, I will explore this difference between TRAK1 and TRAK2 and compare their ability to interact with kinesin using a novel method.

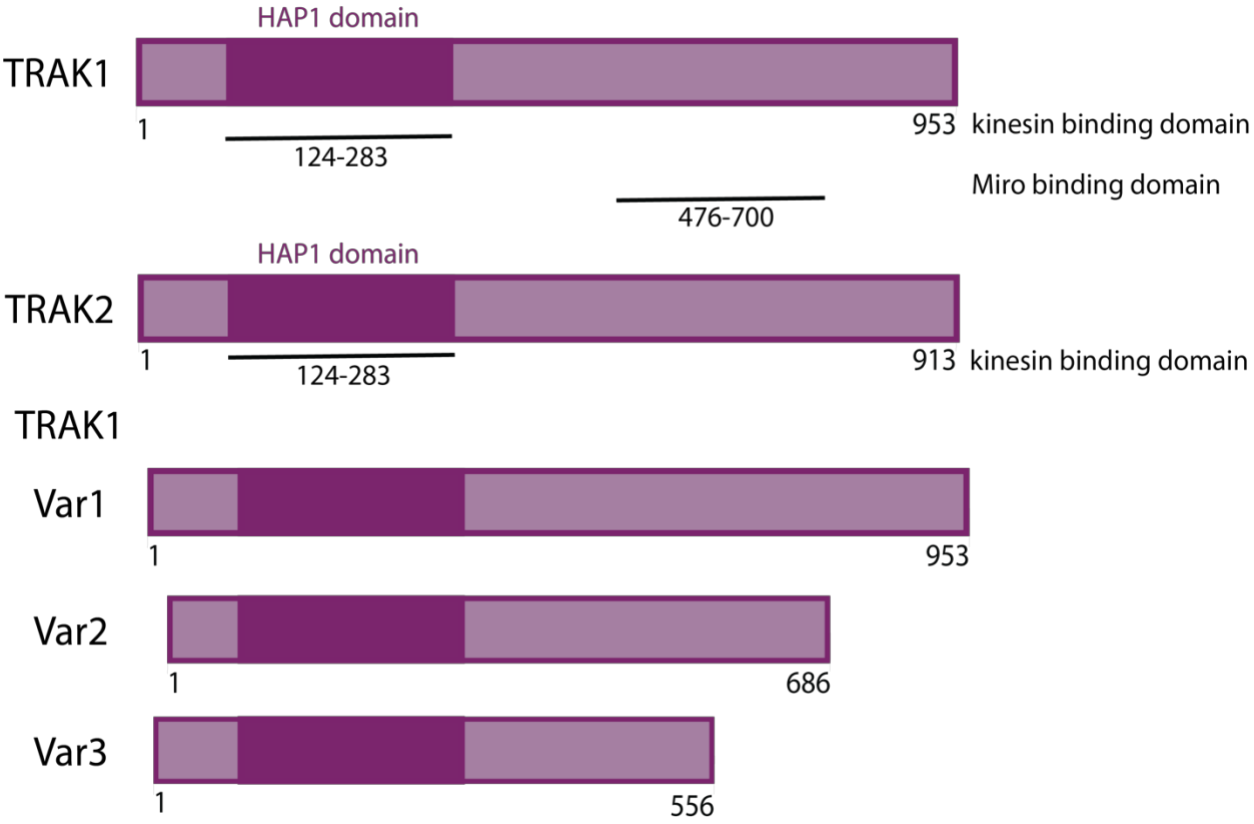


Figure 1.2 Schematic of TRAK1/2 binding domains and variants
Cartoon of TRAK1/2 and TRAK1 splicing isoform variants
TRAK1 interacts with kinesin through its HAP1 domain from 100-360aa. The TRAK1 binding domain with Miro1 is at its C-terminal end from 476-700aa. There are three TRAK1 variants (var-1, var-2, var-3). The TRAK1 splice isoform variants are different lengths with truncations on the C-terminal end of var-2 and var-3.

Miro1/2: the mitochondrial membrane protein

Mammals express two Miro homologs, Miro1 and Miro2, which are 60% identical (figure 1.3). Although Miro1's role in mitochondrial motility has long been studied, Miro2's role in mitochondrial motility is not well understood. There are 4 splicing variants of Miro1 that have

different exons in-between their C-terminal transmembrane domain and the C-terminal GTPase domain. Miro1-var2 and var4 contain an exon 19 region and Miro-var3 and var4 have an exon 20 region (figure 1.3). All Miro1 variants are expressed in both HeLa and HEK293T cells with Miro1-var3 and Miro1-4 found to be expressed at slightly lower levels than var1 and var2. All Miro1 variants were also found to be expressed ubiquitously across tissues (Okumoto et al., 2019). Like Miro1, Miro2 is also found to be ubiquitously expressed across tissues (Quintero et al., 2009). Numerous studies have demonstrated the importance of Miro1 in mitochondrial motility and cellular health. In embryonic neurons, Miro1 knockout leads to a depletion of mitochondria in distal dendrites but not axons, along with a reduction in dendritic complexity. Miro1 knockout in mature neurons leads to fast degeneration of distal dendrites and eventually to cell death (López-Doménech et al., 2016). These results suggest that Miro1 is necessary for the localization of mitochondria in distal dendrites and that this localization is critical for dendritic sustainability. Similarly, studies have found that mice with a constitutive knockout of Miro1 are born alive but die quickly after birth (López-Doménech et al., 2016). In another study of adult mouse neurons, loss of Miro1 led to the depletion of mitochondria from corticospinal tract axons and caused neurological deficits similar to that of human upper motor neuron disease. Although Miro1-deficient adult mouse neurons were shown to have a defect in trafficking, mitochondrial respiratory function and calcium buffering continued as did calcium-mediated regulation of mitochondrial trafficking. (Nguyen et al., 2014).

In contrast, Miro2 knockout mice were viable, with Miro2 knockout having no effect on mitochondrial localization and trafficking in embryonic neurons (López-Doménech et al., 2016). Interestingly, recent studies have shown that mitochondria are still motile in Miro1/2 double knockout cells, although mitochondrial motility is reduced (Kanfer et al., 2015; López-

Doménech et al., 2016). In Miro1/2 double knockout cells, TRAK was reported to localize to the mitochondrial membrane without Miro, and anterograde trafficking was shown to occur; however, retrograde trafficking was shown to be dependent on the presence of Miro1 (López-Doménech et al., 2018). In Drosophila, which has only a single Miro (dMiro) and TRAK (Milton), all mitochondrial transport is dependent on them (Babic et al., 2015). Together, these studies show that Miro and TRAK proteins are necessary for animal development and efficient mitochondrial motility. There are still many open questions about the functional difference between Miro1 and Miro2 with little evidence for the role of Miro2 in the regulation of mitochondrial motility. Although overexpression of Miro1 and Miro2 seem to both localize to all mitochondrial (Fransson et al., 2003), it is not known whether this is true of endogenous Miro1 and Miro 2 or if they localize to different populations of mitochondria. In chapter 2, I investigate the functional differences between Miro1 and Miro2 and build on the current understanding of the role Miro2 plays in mitochondrial trafficking.

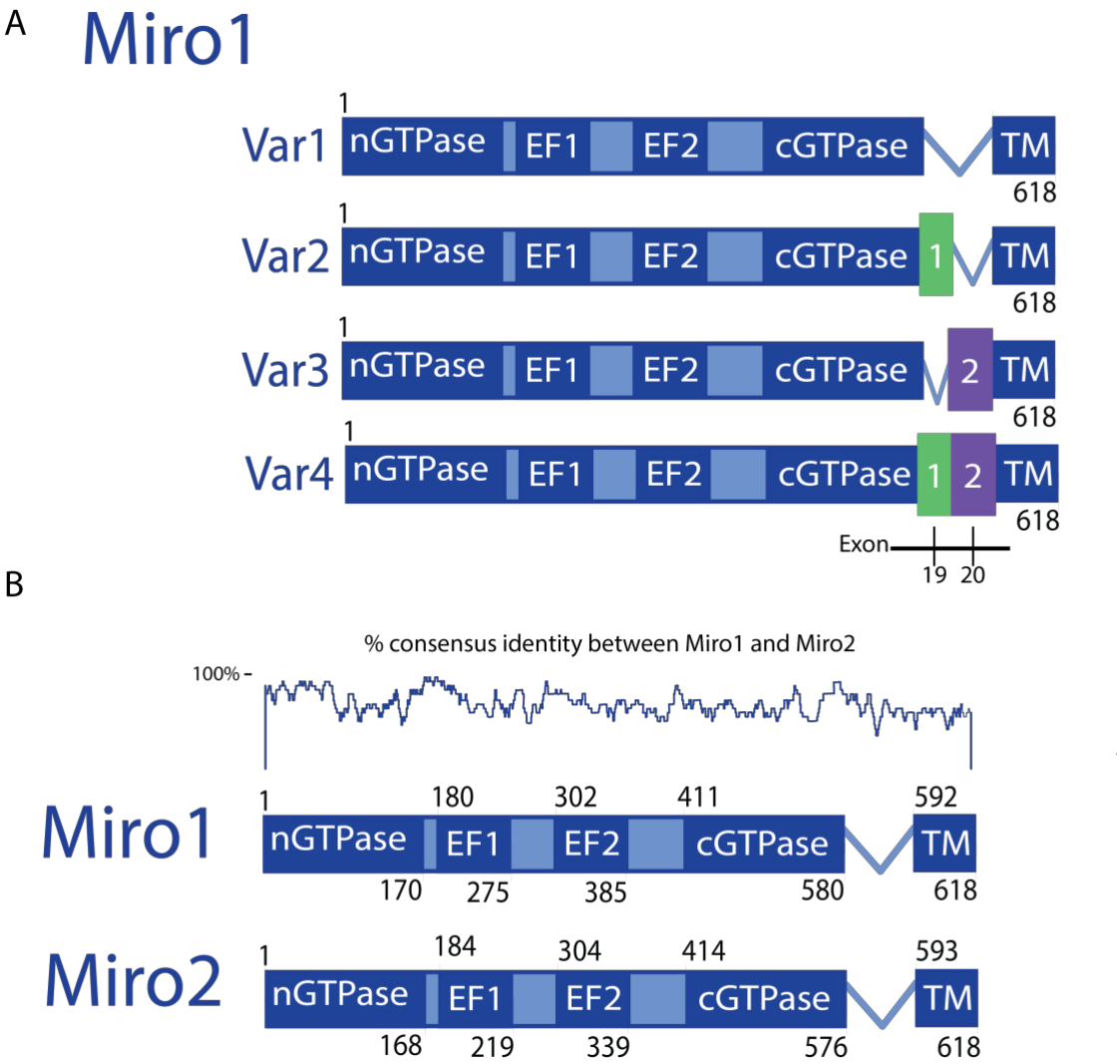


Figure 1.3 Schematic of Miro1/2 binding domains and variants

Cartoon showing Miro1 splicing variants and comparison of Miro1 and Miro2.

A) There are four Miro1 splice variants, var1, var2, var3, and var4. Miro1 var2 and var 4 contain exon 19. Miro1 var3 and var4 contain exon 20.

B) Miro1 and Miro2 are 60% identical.

Regulation of the motor-adaptor complex in mitochondrial trafficking

O-GlycNAcylation of TRAK1

One way to control mitochondrial motility is through the regulation of components of the motor-adaptor complex. An example of this type of regulation is the O-GlcNAcylation of the motor-adaptor TRAK1. Increases in intracellular glucose have been shown to halt mitochondrial trafficking (Pekkurnaz et al., 2014). Through the Hexosamine Biosynthetic Pathway, glucose is

converted to UDP-N- acetylglucosamine (UDP-GlcNAc). Cellular levels of UDP-GlcNAc influence the activity of the enzyme O-GlcNAc Transferase (OGT), which can produce a post-translational modification in a process called O-GlcNAcylation. The TRAK1 motor-adaptor is post-translationally modified by OGT, leading to a suppression in mitochondrial motility (Pekkurnaz et al., 2014). This disruption in mitochondrial motility is an effect of TRAK1 interacting with the protein FHL2 in an OGT-dependent manner. The interaction with FHL2 leads to the association of mitochondria with actin, where OGT-dependent stopped mitochondria become localized to actin filament baskets that physically anchor down mitochondria (Basu et al., *in preparation*).

Calcium regulation of Miro1 EF-hands

Another way that mitochondrial trafficking is regulated through the motor-adaptor complex is through the regulation of the two pairs of Miro1 EF-hands (Smith et al., 2019). Miro is an atypical Rho-like GTPase with tandem GTP-binding domains (nGTPase and cGTPase), which flank either side of the two pairs of calcium-binding EF-hands. Miro has a C-terminal transmembrane domain anchor which positions the protein on the mitochondrial outer membrane. Miro's domain architecture is highly conserved throughout eukaryotes (Fransson et al., 2003; Guo et al., 2005; Klosowiak et al., 2013; Klosowiak et al., 2016). Miro proteins act as Ca^{2+} sensors that regulate Ca^{2+} dependent mitochondrial stopping in both axons and dendrites (MacAskill et al., 2009; Saotome et al., 2008; Wang et al., 2009). The Miro1 EF-hands have been shown to mediate the Ca^{2+}-dependent arrest of mitochondrial movement, where mitochondrial trafficking ceases in the presence of increased cellular Ca^{2+} concentration. Miro1 EF-hand mutations at amino acids 208 and 328 to lysine disrupt the function of the EF-hands (referred to as Miro1^{KK}) by making Miro1 unable to bind Ca^{2+} (Wang et al., 2009). It is unknown

whether Ca^{2+} dependent arrest is solely dependent on the mitochondrial motor-adaptor complex or requires other mitochondrial components. Several different models for Ca^{2+} initiated disruption in mitochondrial trafficking have been proposed. All models agree that the EF-hands of Miro mediate Ca^{2+} dependent stopping. While one group's model for the disruption of mitochondrial trafficking hypothesizes that the kinesin motor falls off the complex, the Schwarz lab has shown a mechanism that inhibits kinesin's motor activity without dissociating kinesin from the motor-adaptor complex. A third group has invoked a requirement for an additional protein, syntaphilin, which tethers the mitochondria to microtubules after Ca^{2+} dependent immobilization (MacAskill et al., 2009; Wang et al., 2009; Chen and Sheng, 2009).

Regulation of mitochondrial trafficking by anchor proteins

Mitochondrial motility is not only regulated by microtubule and actin-based motors but also proteins that act as anchors, binding mitochondria to microtubules or actin filaments in a way that inhibits them from anterograde or retrograde motility. One mechanism of mitochondrial anchoring is regulated by the protein syntaphilin (SNPH). SNPH has been shown to halt mitochondrial motility in axons when localized to mitochondria both by endogenous localization and overexpression. In addition, knockout of SNPH incurs an increased mitochondrial trafficking phenotype (Kang et al., 2007). SNPH has been shown to interact with the KIF5 motor protein directly which has been shown to inhibit kinesin motor-stepping by blocking ATP hydrolysis (Chen & Sheng, 2009). The association between SNPH and the motor-adaptor complex has been reported to be regulated by increased neuronal Ca^{2+} and interaction between Ca^{2+} and Miro1 EF-hands, but this reported mechanism of SNPH recruitment to the motor-adaptor complex is open to question, with several models for Ca^{2+} induced mitochondrial stopping existing in the literature (Chen & Sheng, 2009; Wang et al., 2009).

SNPH is also reported to anchor mitochondria following a ubiquitination event by the E3 ubiquitin ligase CHIP, where SNPH ubiquitination results in SNPH anchoring to tubulin where it inhibits both mitochondrial trafficking and fission and fusion of mitochondria (Seo et al., 2018). Inhibition of SNPH ubiquitination by site-directed mutagenesis led to an increase in the recruitment of the fission regulatory protein Drp1 to mitochondria, revealing a larger role for SNPH in the regulation of mitochondrial dynamics (Seo et al., 2018). With several conflicting mechanisms for SNPH in the literature, more studies and new tools are needed to fully understand when SNPH regulates mitochondrial motility and exactly how it interacts with the mitochondrial motor-adaptor complex.

It has been proposed that intramitochondrial Ca^{2+} concentration could also be regulating mitochondrial transport. Chang et al., 2011 have shown that as intracellular Ca^{2+} concentrations increase, mitochondrial velocity in axons slows. Altogether, calcium is found to be important for the regulation of mitochondrial motility and suggests that the regulation of Miro1 protein's domains is important to study further. In Chapter 3, I attempt to resolve the differences in the three models for Ca^{2+}-dependent regulation of mitochondrial motility and look for a direct association of SNPH with the motor-adaptor complex.

Acetylation of Miro1

In addition to the regulation of Miro by Ca^{2+}, one recent study has identified that Miro1 is regulated by acetylation. The histone deacetylase 6 (HDAC6) deacetylates Miro, leading to an inhibition of mitochondrial transport, suggesting that in order for Miro directed mitochondrial trafficking to occur, Miro must be acetylated (Kalinksi et al., 2019). Human Miro has been found to be acetylated at three different sites, K92, K512, and K616 (Choudhary et al., 2009). In rats, Miro1 acetylation at lysine 105 (human K92) was found to be the critical acetylation site for the

regulation of mitochondrial transport (Kalinksi et al., 2019). This is an exciting result with many open questions remaining, including how acetylation of Miro regulates mitochondrial trafficking and when this event might occur. The acetylation of Miro1 is not addressed in this thesis but will be important to consider for the continuation of this work.

Miro GTPase domains: an in-depth look at structure and function

Miro has two GTPase domains and is recognized as a member of the Ras GTPase family. Typically, GTPases in this family act like molecular switches which cycle between two states, an active GTP-bound state and an inactive GDP-bound state (Syrovatkina et al., 2016; Peters et al., 2018). Like similar GTPases, Miro proteins have conserved GTPase domain G1, G4, and G5 loop sequence motifs. Although the Miro GTPases share many similarities with other small Ras GTPases, they also have unique sequences in their Switch I/II regions and are considered to make up their own GTPase protein family (Peters et al., 2018). The similarities between Miro GTPase domains and other small protein GTPase domains have, for a long time, led scientists to hypothesize that the Miro N-terminal and C-terminal GTPase domains likely act as regulators of Miro activity with potential implications for regulation of the mitochondrial motor-adaptor complex and mitochondrial trafficking (Eberhardt et al., 2020).

Structural studies of Miro1 have attempted to crystalize the full Miro1 protein without success but have managed to separately crystalize the nGTPase of Miro1 in one study and the EF-hand pairs and cGTPase region of Miro1 in a second study (Klosowiak et al., 2013; Smith et al., 2019). Crystallization of the nGTPase of Miro1 found that GTP was bound to the active site, despite not purifying the nGTPase in the presence of additional GTP (Smith et al., 2019). This finding that the Miro1 nGTPase is bound to GTP was in a Miro1 truncation mutant, which

suggests that the nGTPase of Miro1 alone may not be sufficient for the hydrolyzation of the bound GTP (Smith et al., 2019). Structural studies of Miro1 have shown that the cGTPase forms a tight interaction with Miro1's EF-hands, and that the Miro1 cGTPase can bind both GDP and GMPPCP (Klosowiak et al., 2013; Klowsowiak et al., 2016). These findings suggest that the Miro1 nGTPase and cGTPase are functional GTPase domains. As techniques improve, it will be interesting to see the structure of the full Miro1 protein to elucidate whether the GTPase domains might interact with each other or other components of the Miro1 protein that were not crystalized in the truncation mutants. The purification of the full Miro1 protein has also proven to be difficult. Just recently a study was able to purify the full length Miro1 protein that interacts with TRAK1 (Henrichs et al., 2020). It will be interesting to see how this purified Miro1 can be utilized to study the function of the Miro GTPase domains, and I look forward to studies which can show that the Miro GTPase domains facilitate hydrolyzation of the bound GTP.

Previous attempts to characterize the nGTPase and cGTPase domains of Miro have done so by introducing characteristic point mutations within the GTPase domains, which, based on studies of other GTPases, were predicted to alter the Miro GTPase domains into either a GTP-bound constitutively active state or a GDP-bound constitutively inactive state. The Miro1 nGTPase constitutively active GTP-bound mutant is predicted to be P13V (Fransson et al., 2003). Conversely, the nGTPase constitutively inactive GDP-bound mutation is predicted to be T18N (Fransson et al., 2003). In the Miro1 cGTPase, the GTP and GDP- bound like mutants are constitutively active with the K427V mutation or constitutively inactive with the S432N mutation (Fransson et al., 2003; Fransson et al., 2006; MacAskill et al., 2008). In Miro2, the nGTPase has the same T18N constitutively inactive mutation as Miro1 and becomes constitutively active with the P13V mutation. The cGTPase of Miro2 is slightly different from

Miro1 with a S430N constitutively inactive cGTP mutation and a K425V constitutively active mutation (Fransson et al., 2006). Although the literature refers to these mutants as being constitutively active or inactive, it is important to note that the mutations are intended to mimic the state of the GTPase domain protein conformation if GTP or GDP were to be bound to the active site. The constitutively inactive GTPase mutation is intended to alter the protein conformation to a state where GTP can no longer bind to the active site. There is currently no evidence that these mutations actually change GTP or GDP binding to the GTPase domains, but structural studies have agreed with the predicted mutations. Additionally, there is evidence that the GTPase mutants change the function of the Miro1 protein, indicating at the very least that the Miro1 GTPase domains are important for regulating Miro1.

Studying the function of the Miro GTPase domains

Since Miro was first characterized as having two GTPase domains, there have been a number of attempts to understand the function of these domains. Overexpression of Miro constructs, which contain either the constitutively active or constitutively inactive GTPase point mutations, have found that Miro's nGTPase domain is important for regulation of mitochondria (Fransson et al., 2006; Kanfer et al., 2015; MacAskill et al., 2008). In COS-7 cells, overexpression of the N-terminal GTP constitutively bound mutation has been shown to redistribute organelles leading to a perinuclear assembly of mitochondria (Fransson et al., 2003; Fransson et al., 2006). This nGTPase constitutively active form of Miro1 has also been found to trigger apoptosis. In contrast the nGTPase GDP constitutively inactive mutation led to a collapsed mitochondrial network (Fransson et al., 2003). In neurons, the overexpression of the nGTPase constitutively inactive mutant causes a loss of TRAK1 localization to mitochondria and a decrease in mitochondrial trafficking (MacAskill et al., 2008). These results were obtained using the overexpression of

Miro1 mutants, and they did not account for the presence of endogenous Miro1 or Miro2, confounding their conclusions.

In Drosophila, in a Miro loss of function mutant, the nGTPase constitutively inactive mutation led to an accumulation of mitochondria in the soma of neurons and ultimately led to a premature lethality, arresting development at the pupal stage. The constitutively inactive nGTPase mutation also led to decreased anterograde and retrograde motility as well as a fragmented mitochondrial phenotype. In contrast the cGTPase constitutively inactive mutation led to a decrease in only dynein dependent retrograde motility (Babic et al., 2015).

In Miro1/Miro2 double knockout MEFs, mitochondrial trafficking is decreased. The overexpression of wild-type Miro1 can rescue this decrease in motility. When the Miro1 nGTPase constitutively active mutant is overexpressed, it's able to partially rescue mitochondrial motility but only in the anterograde direction. When the nGTPase constitutive inactive Miro mutant is overexpressed it's unable to rescue mitochondrial motility (Norkett et al., 2016). Interestingly in this same study, Miro1 is found to interact with DISC1. Overexpression of the Miro1 nGTPase mutants in Miro double knockout cells found that the Miro1 nGTPase was regulating the interaction between Miro1 and DISC1, with the constitutively active Miro1 nGTPase mutant interacting with DISC1 and the inactive nGTPase mutant not interacting with DISC1 (Norkett et al., 2016). Although this GTPase dependent regulation of the interaction between DISC1 and Miro1 is interesting, the reason for DISC1 interaction with Miro1 is still unknown and much more work will need to be done to fully understand how the Miro1 GTPase plays a role in this interaction.

Together, these studies suggest that the nGTPase GTP or GDP bound state of Miro1 plays a role in distribution and morphology of mitochondria, however; they do not reveal a mechanism for

how the Miro1 GTPase domains regulate mitochondrial trafficking and they leave open a number of questions about whether the differences in mitochondrial distribution and trafficking are a result of a direct change in the mitochondrial motor-adaptor complex or a secondary effect of another function of Miro1. They also fail to address what the functional role of the Miro1 cGTPase is as well as the function of the Miro2 GTPase domains. In Chapter 2, I will address many of these open questions and reveal a mechanism for how the Miro1 nGTPase regulates mitochondrial trafficking.

Regulation of Miro GTPase domains

GTPase proteins are often regulated by GTPase-activating proteins (GAPs) and Guanine-nucleotide exchange factors (GEFs). To date, VopE, a *Vibrio Cholera* Type III secretion system effector, has been described as a potential GAP for Miro, and two different GEFs, GBF1 and Vimar (RAP1GDS1), have been suggested to be potential regulators of Miro1 GTPase domains (Ding et al., 2016; Suzuki et al., 2014; Walch et al., 2018).

While studying *Vibrio cholerae* AM-19226, Suzuki et al., 2014 noticed that in response to infection with Vibro cholerae AM-19226, the mitochondrial network collapsed, leading to a perinuclear clustering phenotype. Interestingly, they discovered that a Type 3 Secretion System (T3SS)-delivered effector, named VopE can prevent this mitochondrial perinuclear clustering phenotype. In addition, they found that VopE directly interacts with both Miro1 and Miro2, preventing the mitochondrial perinuclear localization phenotype and also inhibiting Mfn1-dependent mitochondrial fusion. Using, pull-down assays with Miro1 truncation mutants, they found that VopE binds to the Miro1 nGTPase domain. They also performed a GAP assay by measuring GTP hydrolysis in the Miro1 nGTPase domain in the presence or absence of VopE and found that Miro1 nGTPase activity is enhanced 5-fold in the presence of VopE. They further

proved this by showing that Miro1 GTPase activity could be altered in a VopE dose dependent manner (Suzuki et al., 2014). These results suggest that Miro1 has a functional nGTPase domain which can regulate mitochondrial trafficking and function in a GTPase-dependent manner. Although this result is incredibly interesting, it's only been shown in this unique situation of pathogen infection and it's unlikely that the VopE GTPase activating protein is the only GAP that can regulate Miro. To date, no one has been able to identify an endogenous GTPase-activating protein which can regulate Miro1 and further work will be needed to understand how Miro1 might be regulated by GAPs in more typical scenarios than pathogen infection. Further, there has been no described mechanism for how Miro1 nGTPase interaction with VopE can alter the mitochondrial clustering phenotype. In Chapter 2, I show a mechanism for Miro1 nGTPase regulation that could fit into this model of GTPase dependent rearrangement of mitochondria and in Chapter 4 I will discuss how these models might relate.

In contrast to the thorough work describing VopE as a GTPase-activating protein, the studies identifying Miro1 GEF candidates lack evidence showing that the suggested GEF-dependent phenotypes are indeed GTPase dependent. Vimar, which is the *Drosophila* homolog of RAP1GDS1, was identified as a potential Miro GEF due to its ability to alter mitochondrial morphology phenotypes in a loss of function *Drosophila* mutant and its ability to rescue damaged mitochondrial phenotypes when knocked down in mammalian cells (Ding et al., 2016). In mammalian cells, treated with a calcium ionophore, that leads to mitochondrial fragmentation, RAP1GDS1 knockdown by shRNA rescued mitochondria from fragmentation, rescuing an increased cell death phenotype in comparison to control. RAP1GDS1 has been shown to interact with Miro1 through co-immunoprecipitation but evidence that RAP1GDS1 is interacting with Miro1 through it's GTPase domain or in a GTPase dependent manner is lacking (Ding et al.,

2016). This said, it is interesting that a GEF is able to alter mitochondrial morphology and thus further investigation of the relationship between Miro1 and RAP1GDS1 is warranted.

Like RAP1GDS1, the GEF GBF1 has also been shown to regulate mitochondrial morphology. GBF1 has previously been identified as a GEF for the Arf1 GTPase protein which has been shown to regulate Golgi structure and function (Gillingham et al., 2007). While studying GBF1 function, Walch et al., 2018 observed that GBF1 inhibitors altered mitochondrial network positioning, leading to a collapsed mitochondrial network phenotype and increasing retrograde motility of mitochondria in RPE1 cells. The report then shows that GBF1 interacts with Miro2 through affinity purification and mass spectrometry and that overexpressed GBF1 can co-immunoprecipitate overexpressed Miro1 and Miro2. Although it is interesting that GBF1 interacts with Miro1 and Miro2 and regulates mitochondrial localization, much more evidence is needed to show that GBF1 interacts with Miro1 in a GTPase-dependent manner and that GBF1 directly impacts mitochondria trafficking.

Altogether, the proposed GEFs and GAPs offer an interesting explanation for how Miro1 GTPase might be regulating mitochondrial trafficking, but in the case of the proposed GEFs much more work will be needed to connect the observed phenotypes with Miro1 GTPase function. In addition to further studying the proposed GEFs, it will be exciting to continue to search for an endogenous GAP for Miro1 as well as new Miro1 GEF candidates.

Roles for Miro beyond the mitochondria motor-adaptor complex

Although Miro is primarily thought of as the outer mitochondrial membrane protein in the mitochondrial motor-adaptor complex, a number of recent studies have found novel protein interactions with Miro. While some of these novel interactors fit into the regulation of

mitochondrial trafficking others don't seem to be related to mitochondrial trafficking. Here I will give a brief overview of the most well-characterized interactions.

DISC1 interaction with Miro

DISC1 is a protein that has long been associated with major mental illnesses, including schizophrenia and autism (Brandon and Sawa, 2011; Porteous et al., 2011; Norkett et al., 2017). DISC1 has been shown to interact with Miro1, Miro2, TRAK1, and TRAK2. Disrupting the interaction between DISC1 and Miro or TRAK has been shown to disrupt mitochondrial trafficking (Norkett et al., 2016; Ogawa et al., 2013). Although this relationship has long been known, there are still many questions about the function of DISC1 in interaction with the Miro and TRAK proteins that have yet to be elucidated. It is still not understood how DISC1 interacts with the components of the motor-adaptor complex or what function it has other than being critical for efficient mitochondrial trafficking and cell health.

Actin based mitochondrial trafficking

Actin-based motors have been shown to play a role in mitochondrial trafficking, particularly in regions where microtubules are disrupted (Pathak et al., 2010). Unlike long-range transport on microtubules, actin-based transport in metazoan cells is generally thought to be for short-range local transport. The actin motor protein, Myo19, has long been shown to localize to mitochondria and has also been implicated in actin-mediated mitochondrial trafficking, with overexpression significantly increasing mitochondrial velocity (Quintero et al., 2009). Recently, Myo19 has been shown to interact directly with Miro1/2, and an interaction site between Myo19 and Miro1/2 has been discovered (López-Doménech et al., 2018; Oeding et al., 2018; Bocanegra et al., 2019). In COS-7 fibroblast cells, genetic knockout of Myo19 leads to a disrupted

mitochondrial trafficking phenotype that can be partially rescued by the over-expression of either Miro1 or Miro2. Miro1 and Miro2 form complexes with Myo19, recruit Myo19 to mitochondria, and stabilize it at the mitochondrial membrane. Loss of Miro1 has been shown to result in uneven segregation of mitochondria during cell division and a reduced mitosis rate, which can be partially rescued by over-expression of Myo19 (Chung et al., 2016; López-Doménech et al., 2018). The role of Myo19 in mitochondrial trafficking is still broadly not understood, and it is still unknown if Miro ever preferentially interacts with Myo19 or the TRAK1/2 adaptor to mediate actin-based transport or kinesin and dynein driven microtubule-based transport. Although studies have suggested that Myo19 might play a role in mitochondrial localization during mitosis, this phenomenon has not been robustly studied, and there are still many open questions about when Myo19-dependent mitochondrial trafficking occurs.

A role for Miro in mitochondrial fusion

The finding that mitochondria trafficking still occurs in Miro1 and Miro2 knockout cells was surprising and revealed that there are likely additional mechanisms that can facilitate mitochondrial motility (Babic et al., 2015; Kanfer et al., 2015; López-Doménech et al., 2016). Interestingly, in these Miro1/2 double knock out cells, TRAK1/2 and the molecular motors kinesin and dynein have been shown to still associate with mitochondria, and the overexpression of TRAK and kinesin has been shown to drive mitochondria to microtubule plus-ends even in the absence of Miro (López-Doménech et al., 2016; López-Doménech et al., 2018). These findings suggest that there could be an additional mechanism for TRAK1/2 association with mitochondria. The mitofusin proteins (Mfn1 and Mfn2) are candidates for this association, given that they have previously been shown to interact with both Miro and TRAK (Misko et al., 2019; Lee CA et al., 2018). Mitofusins are members of the dynamin GTPase family and have been

shown to impact mitochondrial trafficking, with mitochondria velocity slowing down and mitochondria pauses lasting longer in Mfn2 knockouts (Misko et al., 2019). In addition to this regulation of mitochondrial motility, the interaction between Mfn2 and TRAK1 has been shown to play a role in mitochondrial fusion events (Lee CA et al., 2018). Further, a mutant variant of Mfn1, $Mfn1^{F202L}$, has been shown to alter mitochondrial transport, increasing the percentage of mitochondria trafficking in the retrograde direction (Engelhart & Hoppins, 2019).

In addition to these reported functions of the mitofusin proteins, unpublished work from Gerald Dorn's lab has been shared at scientific conferences and indicated that Mfn2 might play a more direct role in regulating mitochondrial trafficking by regulating the interaction between Miro1 and TRAK1.

There are still many open questions about how and when Mfn2 interacts with the motor-adaptor complex and how Mfn2 can mediate mitochondrial trafficking. It is still not understood whether Mfn2 plays a direct role in mitochondrial trafficking or if the effect of Mfn2 on mitochondrial trafficking is a reflection of how mitochondrial trafficking relates to fission and fusion events. Given that purified Miro1 and TRAK1 have been shown to be sufficient for mitochondrial trafficking, ample evidence will be needed to prove that Mfn2 regulates the interaction between TRAK1 and Miro1 (Henrichs et al., 2020). In Chapter 3, I follow up on the report of Mfn2 regulating the interaction between Miro1 and TRAK.

Miro1 role in mitochondrial turnover

One reason for mitochondria motility arrest is mitochondrial turnover, termed mitophagy, which is critical for cellular health. The breakdown and recycling of components of sick and damaged mitochondria is critical for proper cellular function, and disruptions in this cellular process have

long been linked to the neurological malady, Parkinson's disease (Hsieh et al., 2016; Hseih et al., 2019; Shaltouki et al., 2018). Mitochondrial turnover is regulated by PINK1, a kinase protein translated on the mitochondrial outer membrane where it is continuously degraded. PINK1 has been found to directly associate with Miro (Wang et al., 2011; Weihofen et al., 2009). When mitochondrial damage occurs, the protein LRRK2 is activated and phosphorylates the Miro1 protein (Hsieh et al., 2016). After phosphorylation by LRRK2, Miro1 then becomes a target for phosphorylation by PINK1 at Miro1 residues, S156, T298, and T299. Phosphomimetic mutants of Miro find that the S156E mutation is sufficient for the recruitment of Parkin to Miro, while the phosphomimetic T298 and T299 mutants are found to inhibit the ubiquitination of Miro and the recruitment of Parkin to Miro (Wang et al., 2011; Shlevkov et al., 2016). PINK1 then phosphorylates the protein Parkin, an E3 ubiquitin ligase that ubiquitinates Miro1, signaling the initiation of the targeted degradation of mitochondria (Safiulina et al., 2019). Interestingly, it has been found that the phosphorylation and ubiquitination of Miro1 by PINK1 and Parkin does not disrupt the interactions between the mitochondrial motor-adaptor complex (Wang et al., 2011). Altogether, these many findings suggest that there is still much we do not understand about the role of the motor-adaptor complex in mitochondrial regulation. Although the motor-adaptor complex has been shown to be sufficient for mitochondrial trafficking, it has not been shown to be necessary for trafficking events to occur, with some mitochondrial trafficking existing in both Miro and TRAK knockout models. With the number of proteins involved in the motor-adaptor complex ever expanding, it is important that those in the mitochondrial trafficking field continue to broaden our view of how mitochondrial trafficking is regulated and consider the many different elements that I've described here.

Miro's role in inter-organelle interactions

Miro interaction with the endoplasmic reticulum

Miro's role in mitochondrial-ER contact sites has long been known. In yeast, the protein Gem1p sits on the outer mitochondrial membrane and is part of the Miro GTPase protein family. Gem1p has been shown to regulate mitochondrial contact with the ERMES complex on the ER membrane. When the Gem1p nGTPase is mutated to be locked in a GDP-like formation, the co-localization of Gem1p with the ERMES complex decreases (Kornmann et al., 2011). This regulation of Gemp1 plays a role in mitochondrial docking at the ER membrane, where ER-associated mitochondrial division occurs. In this process, the ER donates membrane to assist in mitochondrial fission (Friedman et al., 2011). Miro1 and Miro2 have been implicated in a broad range of interactions with proteins in the mammalian ER-mitochondrial contact site (Lee et al., 2018; Modi et al., 2019). Most recently, Miro1, Miro2, and TRAK1 have been reported to directly associate with the ER-Mitochondrial Contact Site (Modi et al., 2019). Although Miro has been shown to interact with a number of ER-associated proteins, the field has just begun to understand the extent of the interactions between the ER membrane and mitochondria and how these interactions are regulated.

A role for Miro1 on peroxisomes

Peroxisomes are membranous organelles whose lumen houses many molecules that work to facilitate the breakdown of reactive oxygen species, bile acid synthesis, and lipid metabolism (Fransen et al., 2017). Like, mitochondria, it is essential that peroxisomes can travel to the areas of the cell where they are most needed. Because peroxisomes break down reactive oxygen species that mitochondria produce during mitochondrial respiration, both organelles' coordination is vital for regulating cellular homeostasis, and they are often located near one another. Recently it has been discovered that these organelles might share a similar process for

organelle trafficking with the discovery that two Miro1 splicing variants interact with peroxisome receptor protein, Pex19. This interaction was described to occur through Miro1 exon 19, which is unique to the two Miro1 variants, Miro1-var2 and Miro1-var4, and located between the Miro1 C-terminal transmembrane domain and cGTPase domain. This interaction between Miro1-var2 and Miro1-var4 with Pex19 is shown through localization of the overexpressed Miro1 variants, co-immunoprecipitation of the overexpressed Miro1 variants with Pex19, and through the presence of endogenous Miro1 in peroxisome fractions (Okumoto et al., 2018; Costello et al., 2017). Alternatively, another study found that the overexpression of Miro1-var1, var2, var3, and var4 were all localized to peroxisomes (López-Doménech et al., 2020). Miro1-var1 and Miro1-var3 do not contain exon 19, but var3 does contain an exon 20 region that's unique to Miro1-var3 and var4. A third study found that overexpression of myc-tagged Miro1-var3 localized to peroxisomes and attributed the difference of localization from the Okumoto report to be due to a difference in cell types (Castro et al., 2018).

In a structure-function experiment, Miro1 ΔnGTPase, which is the deletion mutant of nGTPase, shows an increased localization on peroxisomes, while the nGTPase only truncation mutant did not localize with peroxisomes any more than the full-length Miro1 control. The study suggests that Miro1 interaction with peroxisomes is somehow associated with Miro1 nGTPase, and that the nGTPase domain somehow negatively regulates Miro1's ability to interact with Pex19. The study concludes that Miro1 recruitment to peroxisome membrane is regulated by exon19, exon 20, the C-terminal transmembrane domain of Miro1, and the nGTPase (Covill-Cooke et al., 2020).

In a Miro1/2 double knockout MEF cell, long-range trafficking of peroxisomes is not affected the same way that long-range mitochondrial trafficking is, but a decrease in short-range

peroxisome trafficking is observed (Covill-Cooke et al., 2020). In comparison, Okumoto et al., 2018 find that knockdown of Miro1 regulates long-distance trafficking of peroxisomes. They also find co-localization between TRAK2 and a peroxisome marker, suggesting that TRAK2 interacts with peroxisomal Miro1.

In a different approach for studying the function of Miro on peroxisomes, Castro et al., 2018 misdirected Miro1-var3 to peroxisomes using a fusion Miro1-PEX26-ALDP construct. They found that this re-localization of Miro1-var3 to peroxisomes drives peroxisome trafficking towards the cell's outer periphery. This increase in peroxisome trafficking is again observed when they overexpress a peroxisomal targeted constitutively active N-terminal GTPase domain mutant of Miro1-var1. In contrast, the constitutively inactive nGTPase mutant left peroxisomes scattered throughout the cell (Castro et al., 2018). The misdirection of Miro1-var 3 to peroxisomes is not a good system for studying the function of Miro1 on peroxisomes. This Miro1 variant is also found on mitochondria, so the function of this peroxisome targeted Miro could be a reflection of the function of mitochondria targeted Miro1-var3.

Although several studies have found that Miro localizes to peroxisomes, these studies do not agree on which variants of Miro are on peroxisomes, and they don't agree with how Miro1 seems to affect peroxisomal trafficking. The Miro based mechanism of peroxisome transport is distinct from the hitchhiking peroxisome trafficking model. In this model, peroxisomes are tethered to endosomes via an endosome associated protein, PxdA (Salogiannis et al., 2016). As scientists continue to find new ways that organelles interact, it is important to remember that there's evidence that peroxisomes can be formed from mitochondrial and ER membrane to form pre-peroxisomes (Sugiura et al., 2017). It's also important to further investigate whether peroxisome

located Miro1 serves a peroxisome-specific purpose or if it might be a byproduct of mitochondrial or ER membrane donated to peroxisome formation.

The PEX3Miro system for studying the mitochondrial motor-adaptor complex

When this thesis began, the tools for studying the function of Miro GTPase domains were very limited, and nearly all data discussing the Miro GTPase domains were confounded with the presence of an endogenous Miro1 and Miro2. At the time, animal models suggested that knockout of Miro1 was detrimental to cellular health and that Miro1 knockout animals were not viable. To study the function of the Miro GTPase domains, we had to be creative. To be able to study the Miro1 GTPase domains, my advisor Thomas Schwarz and colleague Himanish Basu developed a plan to re-direct Miro1 and Miro2 to peroxisomes. The recruitment of Miro to peroxisomes allows for the study of the Miro GTPase binding domain function without threatening the health of mitochondria and the cell because mitochondria will continue to have normal levels of Miro. Recruitment of Miro to peroxisomes also allows for studies that distinguish which aspects of mitochondrial behavior are mediated by the motor-adaptor complex and which involve other mitochondrial components.

At the time, there was no known presence of Miro on peroxisomes and it was hypothesized that peroxisome trafficking was infrequent. To re-direct Miro to peroxisomes, C-terminal transmembrane domain deletion mutants of Miro1 and Miro2 were made. The overexpression of these constructs found the localization of Miro to be completely cytosolic. The Miro1 construct lacking the transmembrane domain was then cloned into a new construct with an FRB domain, part of an inducible FKBP-rapalog-FRB heterodimerization system. A second construct contained the transmembrane domain of peroxisomal membrane protein, PEX3, and the FKBP

domain (figure 1.2). If, in the presence of rapalog, Miro is successfully recruited to peroxisomes and can successfully recruit the components of the mitochondrial motor-adaptor complex, then peroxisome targeted nGTPase and cGTPase Miro1 mutants could be used to study the function of the GTPase domains.

Preliminary data showed that the PEX3-FKBP construct's overexpression caused perinuclear accumulation of PEX3, suggesting that it may not be an ideal strategy. We found that when PEX3-FKBP was overexpressed, it can lead to the aggregation of peroxisomes and the localization of fluorescently tagged PEX3-FKBP to mitochondrial membrane in addition to peroxisomes. The phenomenon of PEX3 being present on mitochondria has been observed and further detailed by other research groups (Sugiura et al., 2017). However, this peroxisome-Miro1 recruitment system sufficed to demonstrate the mislocalization of Miro to peroxisomes and increased peroxisome trafficking (Basu et al., *in preparation*).

Interestingly, TRAK1 and kinesin were immediately recruited to peroxisomes upon addition of rapalog to cell cultures, but the increase in peroxisome motility was only observed upwards of 12 hours after the addition of rapalog, suggesting that an additional component may be necessary for motor-adaptor regulated trafficking to occur. Although PEX3 overexpression was localized to both peroxisomes and mitochondria, we decided to continue to use the PEX3 transmembrane domain for the mislocalization of Miro to peroxisomes. We hypothesized that this localization of PEX3 to mitochondria would not affect our ability to study the Miro GTPase domains' function on the motor-adaptor complex and organelle trafficking, as long as we ensured that we were exclusively studying the localization of the Miro and Miro mutant proteins on peroxisomes.

In an attempt to get around the delayed motility of peroxisomes using the PEX3-Miro1 rapalog system, Himanish designed a constitutively conjugated PEX3-Miro1 construct that expresses a

PEX3 transmembrane domain on the N-terminal end of a transmembrane domain deletion mutant of Miro1. The PEX3 transmembrane domain was placed at the N-terminal end of Miro1 to reflect its typical N-terminal placement for recruiting PEX3 to peroxisome membrane (figure 1.2).

Although this constitutively conjugated PEX3Miro1 system still leads to some PEX3 directed mitochondria localization of the construct, I have found by fluorescence microscopy that mislocalized Miro confers on peroxisomes the ability to recruit components of the motor-adaptor complex and undergo motor-adaptor complex dependent trafficking. In Chapter 2 and Chapter 3, I will show how this PEX3Miro construct has been utilized to study the function of the Miro GTPase domains as well as a functional difference between Miro1 and Miro2 and their ability to interact with motor-adaptor complex-associated proteins.

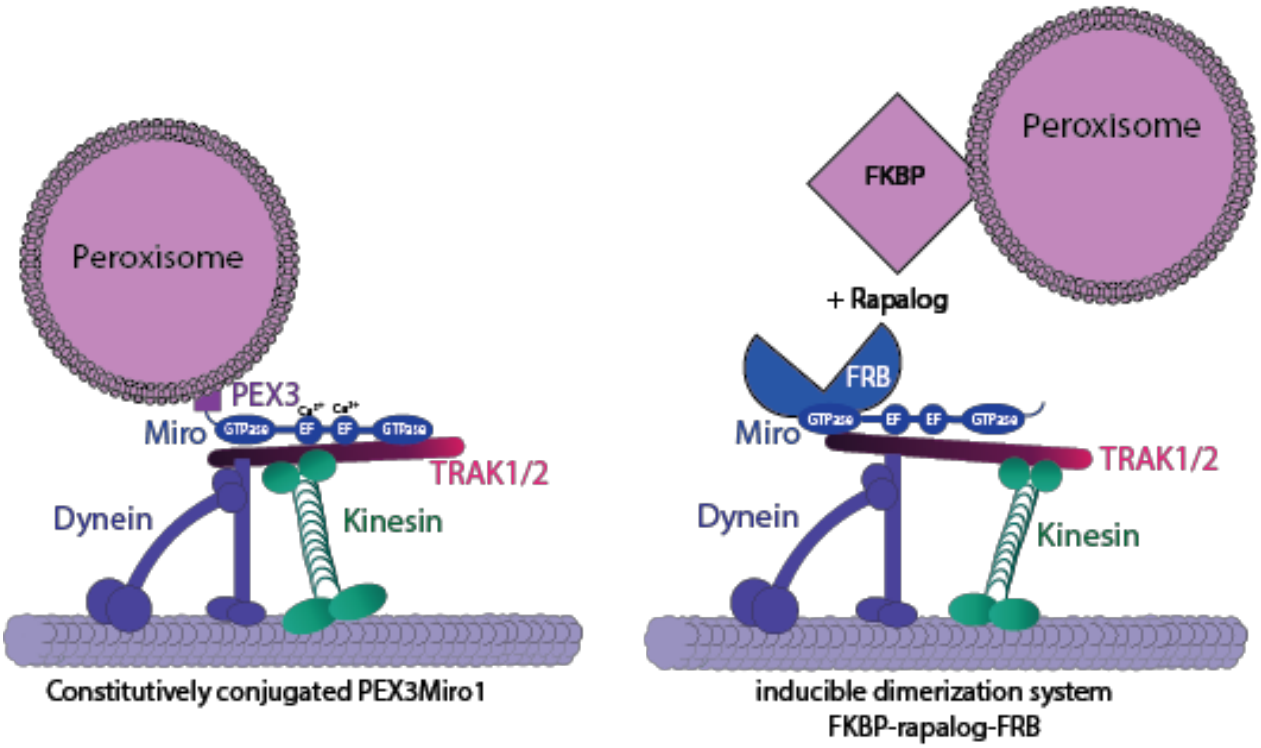


Figure 1.4 Cartoon of the two systems for PEX3 dependent recruitment of Miro to peroxisomes

The constitutively conjugated PEX3Miro construct recruits Miro to peroxisomes via a N-terminally placed PEX3 transmembrane domain. The recruitment of PEX3Miro to peroxisomes enables the recruitment of the motor-adaptor complex to peroxisomes and confers a motor-adaptor complex-dependent increase in peroxisome trafficking. The inducible dimerization system for recruitment of Miro to peroxisomes uses a PEX3-FKBP construct to recruit an FRB-tagged Miro to peroxisomes in the presence of rapalog. This inducible dimerization system is sufficient for the recruitment of Miro to peroxisomes as well as the motor-adaptor complex components, but it has a delayed peroxisome trafficking phenotype.

1.5 Overview of dissertation

During my thesis, I have studied the function of the Miro GTPase domains and their role in regulating the mitochondrial motor-adaptor complex. For this work, I have used a peroxisome-targeted Miro fusion construct, termed PEX3Miro, which has previously been utilized in the Schwarz lab to study TRAK1. I have used the PEX3Miro construct to recruit either wild-type Miro or Miro GTPase domain mutants, which lock the Miro N-terminal and C-terminal GTPase domains in either the constitutively active or constitutively inactive conformation, to peroxisomes. The PEX3Miro system can recruit the motor-adaptor TRAK1/2, and the kinesin-1 motor KIF5C to peroxisomes. Using PEX3Miro, I have found that the N-terminal GTPase domain of Miro1 regulates the motor-adaptor complex assembly, where a Miro1 nGTPase constitutively inactive GDP mutant fails to recruit either the TRAK1/2 adaptor protein or the motor protein KIF5 to peroxisomes. In contrast, the Miro1 nGTPase constitutively active GTP mutant interacts with the motor-adaptor complex but not quite as much as wild-type PEX3Miro. Using the same PEX3Miro1 system, I found that the C-terminal GTPase domain of Miro1 also plays a role in motor-adaptor complex assembly. I have shown how the Miro1 GTPase domains regulate interaction with the motor-adaptor complex through co-localization studies and organelle distribution analyses. Using the PEX3Miro construct, I have found that TRAK1, as opposed to TRAK2, is more efficient at recruiting KIF5 and facilitating plus-end directed motility towards the cell's periphery. I have also used the PEX3Miro system to ask questions about the function of Miro2 in mitochondrial trafficking and found that PEX3Miro2 is not sufficient for recruitment of the motor-adaptor complex to peroxisomes.

During my studies, I have used the PEX3Miro construct to follow up on reports about proteins that interact with the motor-adaptor complex components and have gathered preliminary

evidence to suggest that the motor-adaptor complex does not seem to be sufficient for recruitment of the anchor syntaphilin. I have also used the PEX3Miro system to follow up on reports that MFN2 regulates the interaction between Miro1 and TRAK1. I found that Miro1 interaction with TRAK1 does not seem to be regulated by MFN2, but this result is partially confounded by the discovery that overexpressed MFN2 localizes to peroxisomes. Building on this, I will discuss the PEX3Miro system and several limitations of the PEX3Miro construct that I have discovered throughout my thesis.

Chapter 2
Functional analysis of the Miro GTPase domains and the role of Miro2 in the motor-adaptor complex

Summary

Mitochondrial motility and trafficking are critical for maintaining cellular homeostasis. Mitochondrial trafficking is primarily facilitated by a mitochondrial motor-adaptor protein complex. Here, I report a novel mechanism for regulating mitochondrial motility through the N-terminal GTPase domain of Miro1, the motor-adaptor complex's mitochondrial outer membrane protein. Using a PEX3Miro fusion construct that mislocalizes Miro1 to peroxisomal membrane, we find that the Miro1 N-terminal GTPase domain regulates Miro1's interaction with TRAK1/2, leading to a dissociation of the motor adaptor complex and a decreased peroxisome trafficking phenotype. Using the PEX3Miro system, we find that TRAK1 and TRAK2 co-localize with kinesin, but that TRAK1 co-localizes with kinesin more abundantly than TRAK2. We use the PEX3Miro construct to study the function of PEX3Miro2 and find that PEX3Miro2 does not interact with components of the motor-adaptor complex.

Introduction

Mitochondria are trafficked throughout cells where their distribution is necessary for regulating cellular metabolism and homeostasis. Mitochondrial movements are tightly controlled, with multiple signaling pathways known to regulate their motility (Schwarz, 2013). Mitochondrial trafficking is essential to ensure mitochondria can quickly and efficiently respond to cellular energy demands, calcium buffering, and ROS signaling (Emptage et al., 2001; MacAskill et al., 2009; Marchi et al., 2012; Mattson et al., 2008; Mochinda et al., 2008). The proper distribution of mitochondria is also critical for mitochondrial inheritance during cell division and establishing functional contact sites with other organelles (Chung et al., 2016; Kanfer et al., 2015; Modi et al., 2019). In mammalian cells, mitochondria are trafficked along microtubules by a motor-adaptor protein complex (Brickley et al., 2005; Glater et al., 2006; Hirokawa et al., 1991; Pilling et al.,

2006; van Spronsen et al., 2013; Stowers et al., 2002). When this trafficking is disrupted, cellular homeostasis can be affected, in some cases initiating neurodegeneration, which can lead to a number of degenerative diseases (Hardy, 2010; Liu et al., 2012; Morotz et al., 2012; Nguyen et al., 2014; Wang et al., 2011; Zhang et al., 2015). The motor-adaptor complex comprises Miro, an outer mitochondrial membrane protein and Rho-like GTPase, TRAK, a motor-adaptor protein, and two microtubule-based motors (Brickley et al., 2011; Fransson et al., 2003; Fransson et al., 2006; Glater et al., 2006; Stowers et al., 2002). The molecular motor kinesin-1 (KIF5) transports mitochondria towards the plus-ends of microtubules, and the dynein-dynactin complex transports mitochondria towards the minus-end of microtubules (Drerup et al., 2017; Glater et al., 2006; Hurd and Saxton, 1996; Pillling et al., 2006 Tanaka et al., 1998; Vale et al., 1985). The mitochondrial outer membrane protein, Miro, has been found to participate in many different cellular functions. These functions include mitochondrial turnover, establishing mitochondria-ER contact sites, microtubule-based mitochondrial trafficking, actin-based mitochondrial trafficking, and mitochondrial fission and fusion (Bocanegra et al., 2019; Lee CA et al., 2018; López-Doménech et al., 2018; Misko et al., 2019; Modi et al., 2019; Oeding et al., 2018; Shlevkov et al., 2016; Wang et al., 2011). Some Miro variants have also been found to localize to peroxisomes (Castro et al., 2018; Covill-Cooke et al., 2020; Okumoto et al., 2018). Miro is made up of a C-terminal transmembrane domain that anchors the protein to mitochondria, two GTPase domains, one at the N-terminal end and one at the C-terminal end, and two pairs of EF-hands (Fransson et al., 2003; Klosowiak et al., 2013; Smith et al., 2019).

There are two homologs of Miro, Miro1 and Miro2, which have both been shown to engage in microtubule-based mitochondrial trafficking, actin-based mitochondria trafficking, and the formation of mitochondria-ER contact sites (Fransson et al., 2003; Modi et al., 2019; Oeding et

al., 2018). There are four isoforms of Miro1; var1, var2, var3, and var4. Miro1-var1 and var3 have been found to localize on mitochondria, except in the case of overexpression of Miro1-var1 and var3 where a small amount of Miro1 co-localizes with a peroxisome marker. Miro1-var2 and Miro1-var4 have been shown to localize to peroxisomes through overexpression and organelle fractionation experiments (Costello et al., 2017; Covill-Cooke et al., 2020; Okumoto et al., 2018).

Miro1 is critical for mammalian development, with Miro1 knockout in mice leading to premature death upon birth. Miro1 knockout has also been shown to cause neurodegeneration in embryonic and adult neurons (Babic et al., 2015; López-Doménech et al., 2016; Nguyen et al., 2014). In COS-7 cells, Miro1 knockout causes a decreased mitochondrial trafficking phenotype (López-Doménech et al., 2018). Miro1 has been found to regulate mitochondrial trafficking by Ca^{2+}-dependent regulation of its EF-hands, where an increased intracellular Ca^{2+} flux leaves mitochondria immobile (MacAskill et al., 2009; Saotome et al., 2008; Wang et al., 2009). A number of studies have also found the Miro1 N-terminal GTPase domain to be important for mitochondrial regulation. Previous studies have found that the overexpression of Miro1 point mutations, locking Miro1 nGTPase in a constitutively active state leads to alterations in mitochondrial distribution. Additionally, Miro1 nGTPase mutants that leave the GTPase in a constitutively inactive state cause a decreased trafficking and mitochondria distribution phenotype (Babic et al., 2015; Fransson et al., 2003; Fransson et al., 2006). Although these studies of the GTPase domains imply a regulatory role, the mechanism by which it alters mitochondrial distribution is less clear.

To study the function of Miro GTPase domains without confounding GTPase mutant phenotypes with the presence of endogenous Miro1 and Miro2, we've used a constitutively conjugated

PEX3Miro1 construct, described in Basu et al., *in preparation* that tethers a transmembrane domain deletion mutant of Miro1 Var-3 to peroxisome membrane, and greatly increases the level of Miro on peroxisomes, making the presence of any endogenous peroxisomal Miro irrelevant. We show that this PEX3Miro1 construct is sufficient for the mislocalization of components of the motor-adaptor complex to peroxisomes. Using the PEX3Miro1 construct, we find that the Miro1 nGTPase domain regulates the motor-adaptor complex's assembly. The constitutively inactive Miro1 nGTPase mutant, which cannot bind to GTP, leads to a dissociation of the motor-adaptor complex on peroxisomes as well as a decreased trafficking phenotype. We use the PEX3Miro1 construct to ask several questions about GTPase regulation of the motor-adaptor complex, including a difference in TRAK1/2 dependent co-localization of the kinesin motor. Using a PEX3Miro2 construct, we find that PEX3Miro2 is not sufficient for the mislocalization of the motor-adaptor complex to peroxisomes.

Results

Mislocalization of Miro1 is sufficient for co-localization of the motor-adaptor complex on peroxisomes

To study the function of the Miro1 GTPase domains, we have developed a PEX3Miro1 fusion construct, described in Basu et al., *in preparation*. To make the PEX3Miro1 construct and re-direct Miro1 to peroxisomes, we cleaved the C-terminal transmembrane domain of Miro1 from the Miro1 protein, making the localization of the Miro1 transmembrane deletion construct completely cytosolic. To re-direct Miro1 to peroxisomes, we then fused the transmembrane domain of peroxisome biogenesis factor, PEX3 to the N-terminal end of Miro1. The N-terminal placement of the PEX3 transmembrane domain was selected to match the orientation of the native transmembrane domain in the PEX3 protein. Because Miro1 is typically anchored to

mitochondria through a C-terminal transmembrane domain, we placed an mRFP tag, a 6xhis tag, and an amino acid linker between the N-terminus of Miro1 and the PEX3 transmembrane domain, with a total length of 278 amino acids, resulting in a PEX3-6xhis-mRFP-Miro1 fusion construct. We overexpressed this PEX3Miro1 fusion construct along with a peroxisome marker, mTurquoise-Serine-Arginine-Leucine (SRL). We saw recruitment of the PEX3Miro1 construct to the outer membrane of peroxisomes (figure 2.1A). This successful recruitment of the PEX3Miro1 construct to peroxisomes meant that we could then utilize PEX3Miro1 to attempt to recruit components of the mitochondrial motor-adaptor complex to peroxisomes. If the PEX3Miro1 construct was sufficient for recruitment of components of the mitochondrial motor-adaptor complex to peroxisomes, we could then begin to study the function of the Miro GTPase domains on the assembly of the mitochondrial motor-adaptor complex.

We also made a control construct, PEX3-6xhis-mRFP, which localizes just the transmembrane domain of PEX3, along with a 6xhis tag and an mRFP tag, to peroxisomes. The purpose of this control PEX3 construct is to compare localization of the mitochondrial motor-adaptor complex proteins to peroxisomes in the presence or absence of peroxisomal Miro. While using microscopy to study the co-localization of the PEX3 control and PEX3Miro1 constructs with the mTurquoise-SRL peroxisome marker, a caveat to this PEX3Miro system became apparent. We observed that high overexpression levels of both the PEX3 and PEX3Miro1 construct led to partial localization of the overexpressed fluorescently tagged constructs to what appeared to be mitochondria. This localization was verified with the co-overexpression of both a mitochondrial marker with the PEX3 control or PEX3Miro1 constructs (PEX3 co-localization with mitochondrial marker “mito-GTP” shown in figure 2.1B PEX3Miro1 co-localization with a mitochondrial marker data not shown). At the same time that we were making this observation,

Sugiura et al., 2017 reported that they observed the fluorescent overexpression of a full-length PEX3 protein co-localized with a mitochondrial marker. This result confirmed that the mitochondrial localization of PEX3 and PEX3Miro1 that we observed was at least partially due to the overexpression of the PEX3 transmembrane domain. We found that a lower overexpression amount of the PEX3 and PEX3Miro1 construct minimized mitochondrial localization and therefore only utilized .1ug of PEX3 and PEX3Miro1 DNA in the cellular transfections for all of the COS-7 experiments discussed in this thesis. Unless otherwise noted, for all of the co-localization and imaging experiments in this thesis, we utilized COS-7 fibroblasts which are derived from the kidney of African green monkeys. These cells are relatively large and also relatively flat making them ideal for imaging. This localization of overexpressed PEX3 and PEX3Miro1 to mitochondria called for a careful experimental design that ensured that co-localization experiments utilizing the PEX3Miro1 system only observed construct co-localization on peroxisomes, in isolation from their potential mitochondrial localization.

To meet our goal of studying the peroxisome-localized PEX3Miro1 without the potential of confounding our results with PEX3Miro1 present on mitochondria or the presence of endogenous Miro localized to mitochondria, we co-overexpress PEX3 and PEX3Miro1 constructs with the mTurqoise-SRL marker (unless otherwise noted) in all experiments in this thesis. We then only consider co-localization of PEX3 control or PEX3Miro1 signals and those of other components of the motor-adaptor complex that overlap with the fluorescently tagged peroxisome marker. We control for differences in PEX3 and PEX3Miro1 expression across cells and bio-replicates by keeping imaging conditions consistent. Only cells with visible PEX3 or PEX3Miro1 expression co-localized with the SRL-mTurquoise peroxisome marker are selected

for imaging. To control for expression specific difference in the localization of PEX3 and PEX3Miro1, we standardized our imaging conditions across all experiments, imaging cells with 5% laser power and a gain of 100, which accounts for 1/5 of the total gain power of the microscope. After co-overexpressing the PEX3 and PEX3Miro1 construct with the mTurquoise-SRL peroxisome marker, we found that they both localized to peroxisome membrane (figure 2.1A).

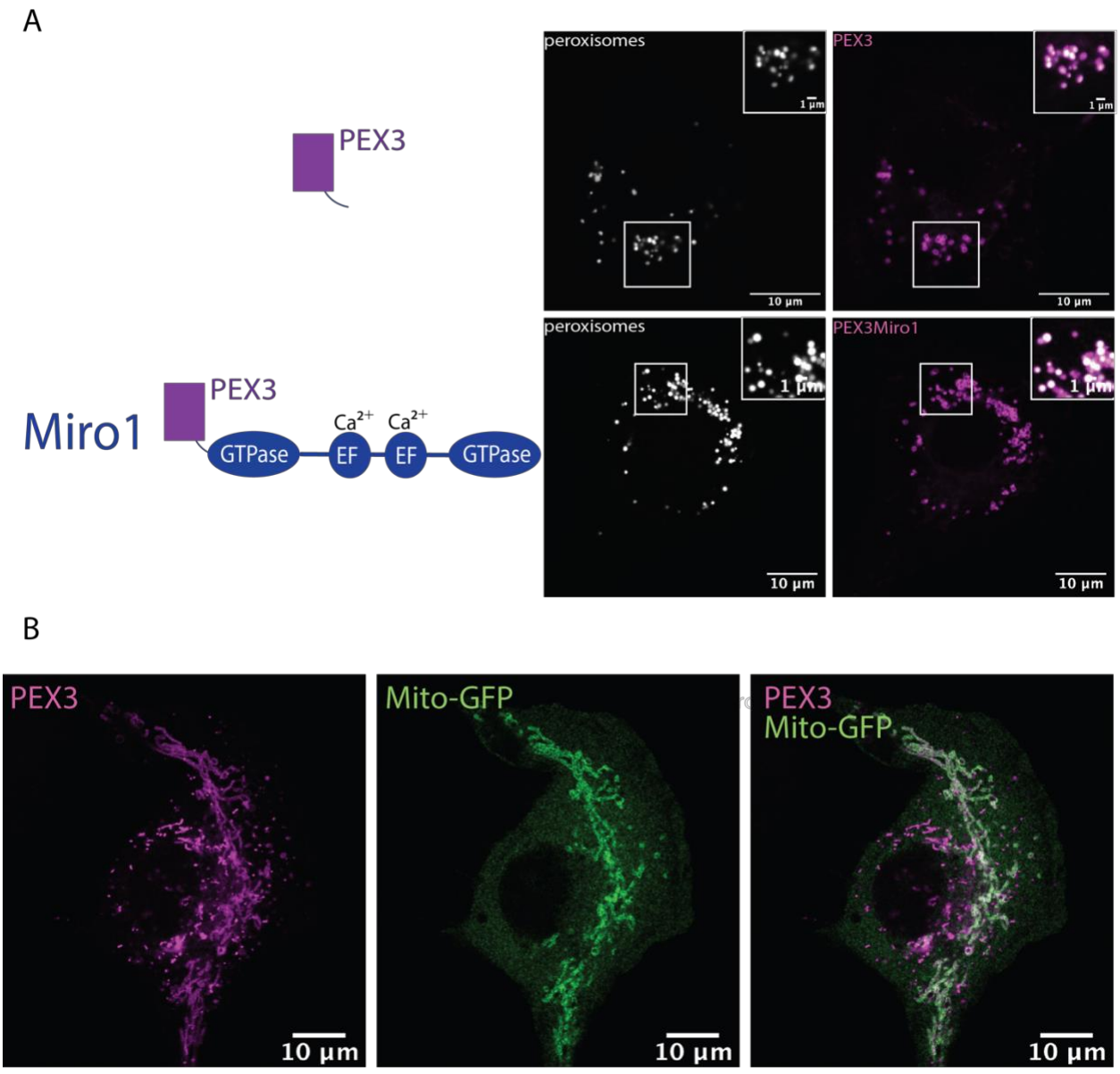


Figure 2.1 PEX3Miro1 localizes to peroxisome membrane

Fluorescently tagged PEX3-6xhis-mRFP transmembrane domain control and PEX3-6xhis-mRFP-Miro1 localize to peroxisome membrane but also to mitochondria at high expression levels

- *A) COS-7 cells were co-transfected with PEX3-mRFP-6xhis (control) or mRFP-6xhis-PEX3Miro1 along with the mTurquoise-SRL peroxisome marker (labeled as "peroxisomes" in white) and incubated for 48 hours before fixation. N=15 over 3 biological replicates.*
- *B) A representative image of high expression levels of the overexpressed PEX3-6xhis-mRFP transmembrane domain control co-localizes with the mitochondrial marker mito-GFP in COS-7 cells fixed and imaged at room temperature after 48-hour incubation following transfection.*

To test whether the PEX3Miro1 construct was sufficient for relocalization of the motor-adaptor complex to peroxisomes, we overexpressed either the PEX3 control or PEX3Miro1 construct with the mTurquoise-SRL peroxisome marker, the mitochondrial motor-adaptor protein myc-TRAK1, and the mitochondrial motor protein mCitrine-KIF5C in COS-7 fibroblasts. KIF5C is a kinesin-1 motor that's only found to be expressed in neurons where it serves as the predominant kinesin motor responsible for mitochondrial transport in axons. COS-7 cells were fixed 48 hours after transfection and imaged. Images were analyzed for co-localization of mCitrine-KIF5C with PEX3 or PEX3Miro1 positive peroxisomes. Peroxisomes were marked using the mTurquoise-SRL marker and only peroxisomes that co-localized with either PEX3 control or PEX3Miro1 construct were analyzed for co-localization with mCitrine-KIF5C. When looking at the co-localization of mCitrine-KIF5C with PEX3 control or PEX3Miro1 positive peroxisomes, we found that peroxisomes with the PEX3 control localized to them did not co-localize with the mCitrine-KIF5C construct in the presence of overexpressed myc-TRAK1. In contrast, peroxisomes that co-localize with the PEX3Miro1 co-localized with mCitrine-KIF5C when myc-TRAK1 was co-overexpressed (figure2.2 A). We conducted a parallel set of experiments that omitted overexpression of myc-Trak1 and found that, in that condition, mCitrine-KIF5C was no longer co-localized with the labelled PEX3Miro1 positive peroxisomes (figure2.2 A).

The co-localization of KIF5C with PEX3Miro1 peroxisomes was quantified using a custom FIJI macro. Images of cells were acquired using z-stacks with .3um thickness and z-slice images were acquired from top to the bottom of cells to account for all peroxisomes. This custom macro creates a mask of the peroxisome marker channel in each z-slice and uses the peroxisome mask to analyze only the pixels in the image where the mTurquoise-SRL peroxisome marker co-localizes with the PEX3 or PEX3Miro1 construct. The PEX3 and PEX3Miro1 positive

peroxisomes are then analyzed for co-localization with overexpressed mCitrine-KIF5C. The final quantification is the enrichment of mCitrine-KIF5C on PEX3 or PEX3Miro1 positive peroxisomes. This macro then controls for any PEX3 or PEX3Miro1 expression-dependent differences in co-localization. Because the expression of the PEX3 control or PEX3Miro1 construct varies slightly from cell to cell, we measured the intensity of the PEX3 control and PEX3Miro1 construct that is localized to peroxisomes as well as the intensity of the mCitrine-KI5C construct localized to peroxisomes. We then control for potential error in co-localization quantification by taking into consideration the partial cytosolic localization of the overexpressed mCitrine-KIF5C. To do this, we subtract the whole cell intensity of the mCitrine-KIF5C signal that exists outside of the peroxisome mask from the specific signal intensity that overlaps with the peroxisome mask. This background fluorescence subtraction allows us to quantify the specific amount of signal on PEX3 control or PEX3Miro1 peroxisomes without cofounding results with the presence of the cytosolic localization of the overexpressed constructs. We further normalize the amount of mCitrine-KIF5C co-localized to peroxisomes with the amount of PEX3 or PEX3Miro1 co-localized with peroxisomes to establish the amount of mCitrine-KIF5C recruited to peroxisomes in a PEX3Miro1 dependent manner.

This macro allows us to quantify peroxisome specific co-localization of PEX3Miro and KIF5C without confounding our results with any PEX3-dependent expression of PEX3Miro on mitochondria. For these experiments and all subsequent co-localization experiments to quantify the co-localization of the mitochondrial motor-adaptor complex proteins with the PEX3 control or PEX3Miro peroxisomes, we employed this same analytical method.

We find that our macro can find a significant difference in the enrichment of mCitrine-KIF5C on PEX3Miro1 peroxisomes in comparison to PEX3 control peroxisomes when co-overexpressed

with myc-TRAK1 (figure 2.2B). Importantly when we quantify the co-localization of mCitrine-KIF5C with the PEX3 control, there is no detectable mCitrine-KIF5C co-localized with peroxisomes, which suggest that even if endogenous Miro1 was present on peroxisomes, there is not a sufficient amount to recruit a detectable level of the overexpressed KIF5C. These findings enable us to use the PEX3Miro system to study the regulation of the motor-adaptor complex without concern about the influence of endogenous wild-type Miro.

Peroxisomes in COS-7 cells are typically localized close to the nucleus. Although they are capable of microtubule-based transport, we've observed that peroxisomes in COS-7 cells do not move much and are never localized to the outer periphery of COS-7 cells. This perinuclear localization of peroxisomes was maintained when we overexpressed the PEX3 control construct which did not affect peroxisome distribution. The overexpression of the PEX3Miro1 construct also did not affect the cellular localization of peroxisomes. When we overexpressed the PEX3Miro1 construct along with the myc-TRAK1 and mCitrine-KIF5C constructs, we observed that peroxisomes had an increased motility, which has been reported in Basu et al., *in preparation.* We also observed a PEX3Miro1 dependent change in the dispersal of peroxisomes where the co-overexpression of PEX3Miro1 with myc-TRAK1 and mCitrine-KIF5C drastically altered peroxisome distribution in COS-7 cells, driving peroxisomes to the outer periphery of the cells (figure 2.2C). In all experiments in this thesis where distribution of peroxisomes is calculated, the mTurquoise-SRL peroxisome marker was overexpressed and used for this quantification. This distribution of peroxisomes was quantified using the DoveSonoPro (Basu et al., 2020) image analysis macro, which is a FIJI based macro. DoveSonoPro measures the % frequency of objects as they appear in concentric shells moving outwards from the center of the cell. The macro takes into consideration the shape of the cell and uses this shape to draw the

shells used to quantify object distribution. Importantly, we found that both the co-localization of KIF5C to peroxisomes and the increased peroxisome distribution phenotype was dependent on the co-overexpression of myc-TRAK1 with KIF5C. These results establish that PEX3Miro1 is sufficient for localizing the motor-adaptor complex to peroxisomes and that PEX3Miro1 peroxisomes, which have successfully mislocalized the motor-adaptor complex, exhibit a motor-adaptor-dependent distribution phenotype. To further confirm that the co-localization of KIF5C with peroxisomes was dependent on the presence of the overexpressed PEX3Miro1, we overexpressed RFP-tagged PEX3 or PEX3Miro1 in HEK233Ts along with mCitrine-KIF5C and myc-TRAK1 and found that myc-TRAK1 and mCitrine-KIF5C co-immunoprecipitated with PEX3Miro1 but not with the PEX3 control (figure 2.2 D).

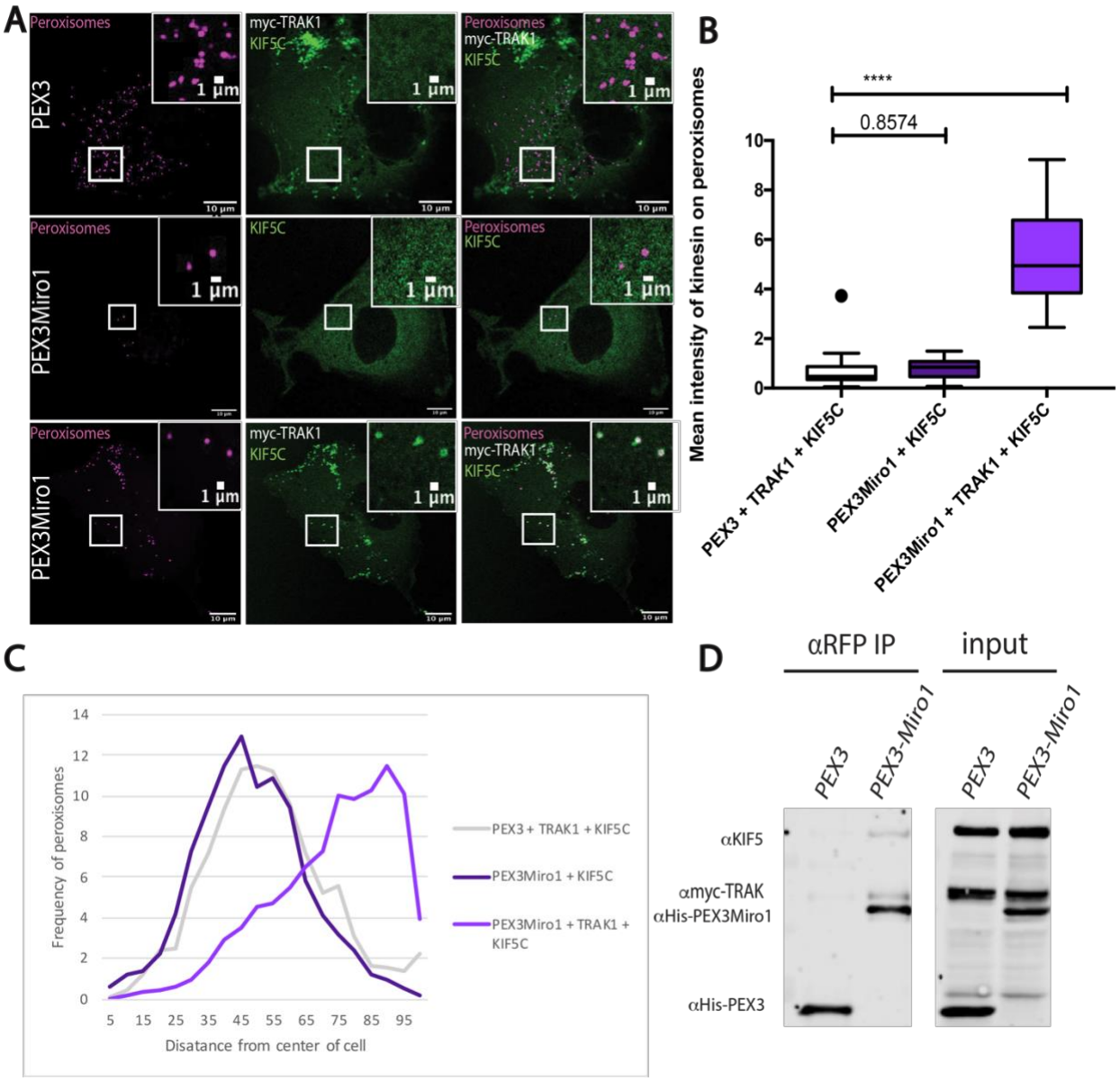


Figure 2.2 PEX3Miro1 can localize KIF5C to peroxisomes with TRAK1 overexpression

The PEX3Miro1 construct is sufficient to mislocalize the motor-adaptor complex to peroxisomes in both COS-7 cells and in HEK293T cell lysates.

A) Overexpression of mTurquoise-SRL, marked as "peroxisomes" in magenta with either 6xhis-mRFP-PEX3 control (signal not shown) or 6xhis-mRFP-PEX3Miro1(signal not shown) with mCitrine-KIF5C in the presence or absence of the overexpression of myc-TRAK1 (signal not shown) in COS-7 cells.

B) Quantification of the amount of KIF5C enriched on PEX3 control or PEX3Miro1 peroxisomes in the presence or absence of the overexpression of myc-TRAK1. The quantifications are represented as 'Box and Whiskers' plots. The line indicates the median. Outliers are represented as individual dots and are considered in all statistical calculations. For demonstrating statistical significance, two-tailed unpaired t-test with Welch correction was used. For comparison of PEX3 to PEX3Miro co-localization with KIF5C p-value is $P < 0.0001$. *There was no statistical difference between PEX3 control and PEX3Miro1 without TRAK1. N=15 cells over 3 biological replicates.*

Figure 2.2 PEX3Miro1 can localize KIF5C to peroxisomes with TRAK1 overexpression cont.

C) Distribution of peroxisomes, marked with mTurquoise-SRL in COS-7 cells with overexpression of 6xhis-mRFP-PEX3 (control) or 6xhis-mRFP-PEX3Miro1 with mCitrine-KIF5C in the presence or absence of the overexpression of myc-TRAK1. Quantified using the DoveSonoPro FIJI macro. N=15 cells over 3 biological replicates.

D) 6xhis-mRFP-PEX3 (control) or 6xhis-mRFP-PEX3Miro1 were overexpressed in HEK293T cells along with myc-TRAK1 and mCitrine-KIF5C and co-immunoprecipitated using the RFP tag on the PEX3 and PEX3Miro1 constructs. Western blots were stained using His, myc, and KIF5 antibodies.

TRAK1 and TRAK2 both associate with Miro1 and localize KIFC to PEX3Miro1

Although the functional difference between TRAK1 and TRAK2 has yet to be fully elucidated, we hypothesized that overexpressing myc-TRAK2 would be sufficient to mislocalize KIF5C to PEX3Miro1 peroxisomes. TRAK2 has previously been shown to be important for motor-adaptor complex dependent mitochondrial motility (López-Doménech et al., 2018, Babic et al., 2015). We found that in the presence of overexpressed TRAK2, KIF5C is localized to PEX3Miro1 positive peroxisomes (figure 2.3 A). Quantification of KIF5C colocalization with PEX3Miro1 peroxisomes, marked with the mTurquoise-SRL peroxisome marker, shows that while TRAK2 is able to localize KIF5C to peroxisomes, it does not localize nearly as much KIF5C motor to PEX3Miro1 peroxisomes as overexpression of TRAK1 does (figures 2.3 B)

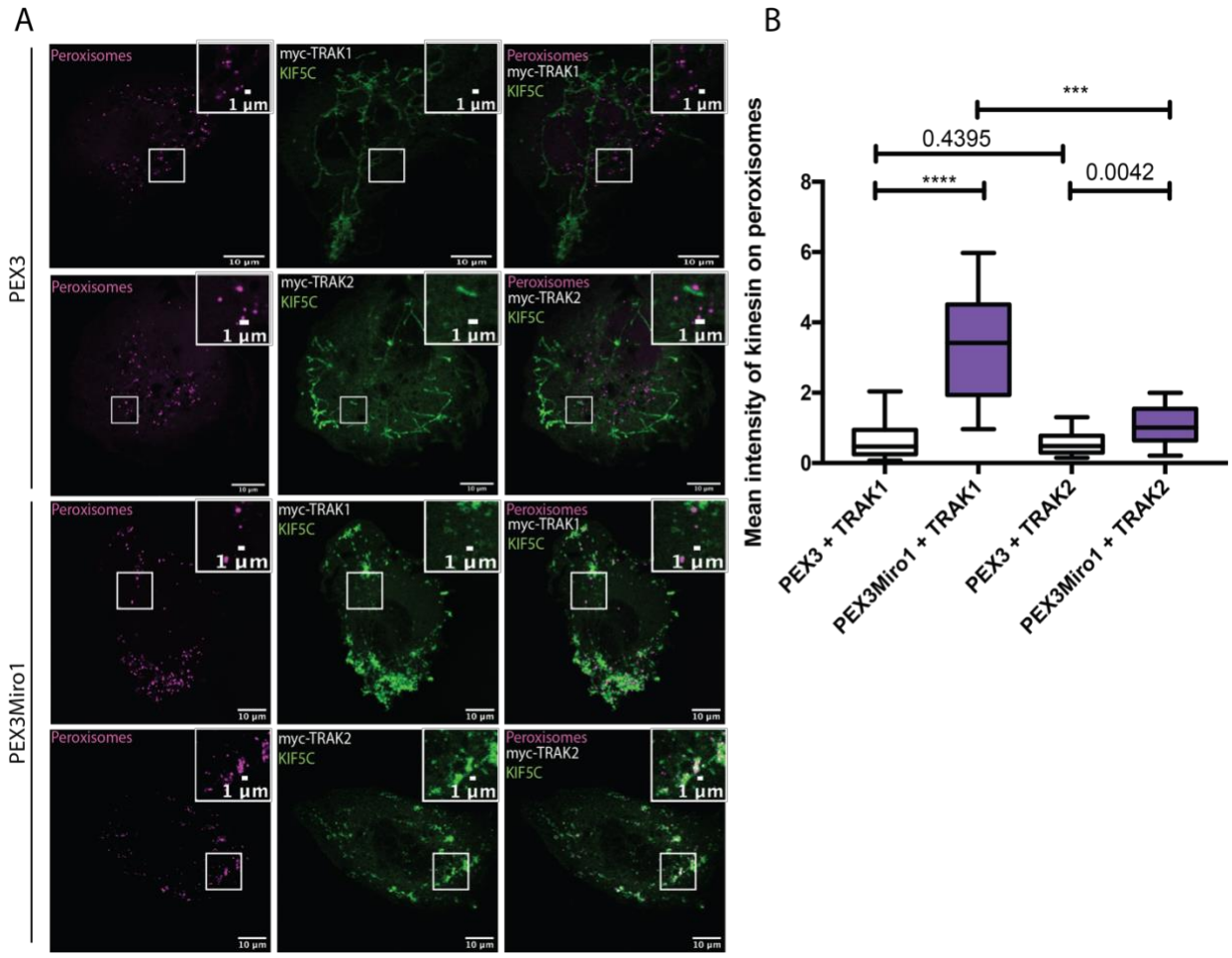


Figure 2.3 TRAK1 and TRAK2 serve as an adaptor for KIF5C

TRAK1 and TRAK2 adaptor proteins are sufficient for the localization of KIF5C to PEX3Miro1 peroxisomes but TRAK1 co-localizes with significantly more KIF5C

- *A) Overexpression of mTurquoise-SRL (marked as "peroxisomes" in magenta) with either 6xhis-mRFP-PEX3 control or 6xhis-mRFP-PEX3Miro1(mRFP signal for PEX3 and PEX3Miro1 not shown) with mCitrine-KIF5C in the presence of the overexpression of myc-TRAK1 or myc-TRAK2 (signal for myc not shown) in COS-7 cells. N=15 cells over 3 biological replicates.*
- *B) Quantification of the amount of KIF5C co-localized with PEX3Miro1 peroxisomes in the presence of myc-TRAK1 or myc-TRAK2 compared to PEX3 control peroxisomes. The quantification is represented as 'Box and Whiskers' plots. The line indicates the median. For demonstrating statistical significance, two-tailed unpaired t-test with Welch correction was used. For comparison of PEX3 to PEX3Miro with myc-TRAK1 co-localization with KIF5C p-value is P <0.0001. There was no statistical difference between PEX3 control and PEX3Miro1 without overexpression of myc-TRAK1. The statistical difference between PEX3 with TRAK1 or TRAK2 is P-value= 0.4395. A statistically significant difference is found between PEX3Miro1 and KIF5C co-localization depending on co-expression with either TRAK1 or TRAK2 P-value 0.0002. N=15 cells over 3 biological replicates.*

Differences in TRAK1 and TRAK2 function have previously been reported. In neurons, TRAK1 serves as the primary adaptor protein for KIF5C driven plus-end directed trafficking and TRAK2 serves as the primary adaptor for dynein driven minus-end directed trafficking (Brickley et al., 2005; MacAskill et al., 2008). Do TRAK1 and TRAK2 differ in their ability to recruit KIF5C or do differences in the amount of TRAK on the peroxisomes explain the differences in the amount of TRAK on the peroxisomes explain the difference in KIF5C recruited? We therefore compared the co-localization of overexpressed mCitrine-TRAK1 and mCitrine-TRAK2 to PEX3Miro1 peroxisomes in COS-7 cells (figure 2.4 A). Quantification of the co-localization of mCitrine-TRAK1/2 with PEX3Miro1 peroxisomes found that a similar amount of mCitrine-TRAK1 and mCitrine-TRAK2 was localized on peroxisomes (figure 2.4B). This result suggests that the difference in KIF5C co-localization with PEX3Miro1 peroxisomes in the presence of myc-TRAK1 or myc-TRAK2 is dependent on their ability to bind KIF5C and not an artifact of TRAK expression differences or the ability of TRAK1/2 to interact with PEX3Miro1.

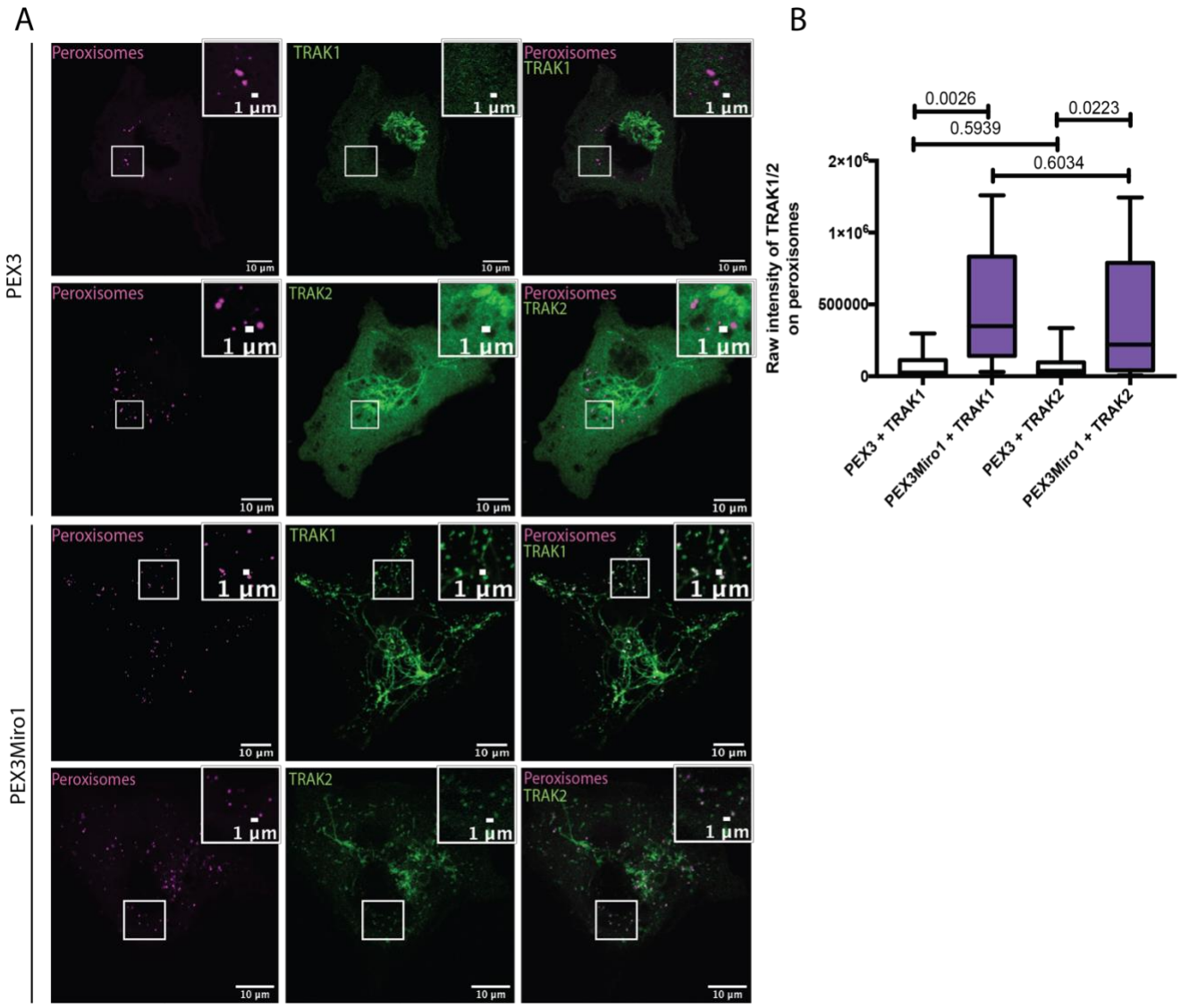


Figure 2.4 A difference in TRAK1/2-dependent localization of KIF5C

Fluorescently tagged mCitrine-TRAK1 and mCitrine-TRAK2 constructs co-localize similarly with PEX3Miro1.

A) Overexpression of mTurquoise-SRL (marked as "peroxisomes" in magenta) with either 6xhis-mRFP-PEX3 or 6xhis-mRFP-PEX3Miro1 (mRFP signal not shown) with mCitrine-tagged TRAK1 or mCitrine-TRAK2 in COS-7 cells. Cells were fixed after 48 hours of expression and imaged at room temperature.

B) Quantification of the raw signal intensity of mCitrine-tagged TRAK1 or mCitrine-TRAK2 co-localized to peroxisomes. The quantification is represented as 'Box and Whiskers' plots. The line indicates the median. For demonstrating statistical significance, two-tailed unpaired t-test with Welch correction was used. N=15 cells over 3 biological replicates

To further ask if the difference in KIF5C colocalization with PEX3Miro1 peroxisomes was dependent on a difference in the TRAK adaptor's ability to localize KIF5C to PEX3Miro1 peroxisomes, we overexpressed PEX3 or PEX3Miro1 with an mturqouise-KIF5C construct in the presence of either mCitrine-TRAK1 or mCitrine-TRAK2 (figure 2.5A). In this experiment we did not overexpress the mTurquoise-SRL peroxisome marker because no other colors were available for microscopy. Quantification of this data is in process and co-localization will be quantified as a measure of the amount of mCitrine-TRAK1/2 and mTurquoise-KIF5C that's co-localized with the PEX3 control or PEX3Miro1 construct without a peroxisome marker.

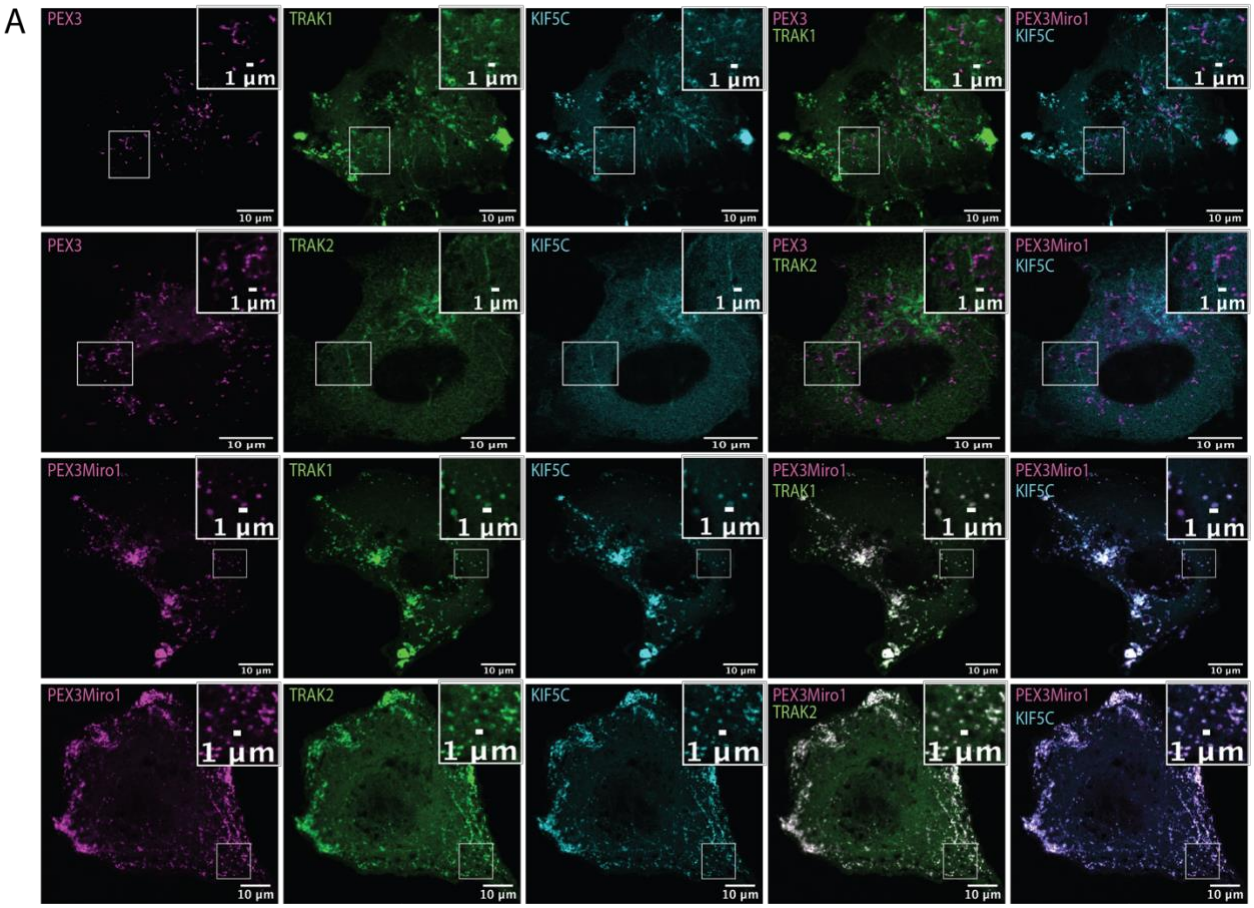


Figure 2.5 TRAK1 and TRAK2 localize to PEX3Miro1 peroxisomes where they co-localize with KIF5C

mCitrine-TRAK1 and mCitrine-TRAK2 co-localize with PEX3Miro1 and mTurquoise-KIF5C in COS-7 cells

A) Overexpression of 6xhis-mRFP-PEX3 or 6xhis-mRFP-PEX3Miro1 (shown in magenta) with mCitrine-tagged TRAK1 or TRAK2 (shown in green) and mTurquoise-KIF5C (shown in blue) in COS-7 cells. PEX3 control does not co-localize with mCitrine-TRAK1/2 or mTurquoise-KIF5C. PEX3Miro1 co-localizes with both mCitrine-TRAK1 and mCitrine-TRAK2 as well as mTurquoise-KIF5C in fixed COS-7 cells 48 hours after transfection. N=15 over 3 biological replicates.

Miro1 N-terminal GTPase regulates interaction with mitochondrial motor-adaptor complex

The successful localization of the motor-adaptor complex to peroxisomes with PEX3Miro1 established a system to test how Miro1 regulates the motor-adaptor complex without confounding the results with the presence of endogenous Miro. This system allows us to investigate the function of the Miro1 nGTPase domains which have long been known to affect mitochondrial motility and cell health, but through an uncertain mechanism. We made single

point mutations to the Miro1 GTPase domains in the PEX3Miro1 construct. These point mutations confer either a constitutively active GTP-bound conformation (nGTPase:P13V or cGTPase:K427N) or constitutively inactive GDP-bound conformation (nGTPase:T18N or cGTPaseS432N) at either the N-terminal or C-terminal Miro1 GTPase domains (Fransson et al., 2006). Although the literature refers to these mutations as constitutively active or inactive, it is important to note that the constitutively active mutation locks the Miro1 nGTPase in a GTP-bound conformation. Crystallization of Miro1 nGTPase found that GTP was bound to the nGTPase (Smith et al., 2019). The inactive Miro1 GTPase mutant locks the GTPase in a predicted GDP-bound conformation where GTP should no longer be able to bind. Mutations locking the nGTPase in the GDP-bound conformation altered mitochondrial motility and also prevented Myo19 and DISC1 interactions with Miro (Norkett et al., 2016; Oeding et al., 2018). In structural studies, the cGTPase domain has been found to bind to GDP, GDP-Pi, and GMPPCP, indicating it also likely functions as an active GTPase domain. Functional studies, however, have yet to elucidate a cGTPase dependent function (Klosowiak et al., 2013).

To determine whether the nGTPase domain regulates the assembly of the motor-adaptor complex, we compared the ability of wild-type PEX3Miro1 to localize the motor-adaptor complex to peroxisomes to that of the nGTPase GTP and GDP mutants. We found that when the N-terminal GTPase is in the inactive GDP mutation, PEX3Miro1 can no longer localize KIF5C to PEX3Miro1 peroxisomes. In contrast, the constitutively active GTP mutation can localize KIF5C to PEX3Miro1 peroxisomes (figure 2.6A). The distribution of peroxisomes also reflects the differences in KIF5C recruitment; peroxisomes move to the periphery in cells expressing wild-type or GTP-state mutant PEX3Miro1 but not in cells expressing the GDP-state mutant or the PEX3 control (Figure 2.6B. When we quantified the co-localization of KIF5C on

PEX3Miro1 peroxisomes using the previously described method, we were surprised to find that the constitutively active GTP mutant does not co-localize with a similar amount of KIF5C as wild-type PEX3Miro1. This difference in co-localization suggests that the nGTPase constitutively active mutation is not alone sufficient to efficiently localize KIF5C to PEX3Miro1 peroxisomes and that the constitutively active GTP mutation may not be equivalent to wild-type PEX3Miro1 with a bound GTP.

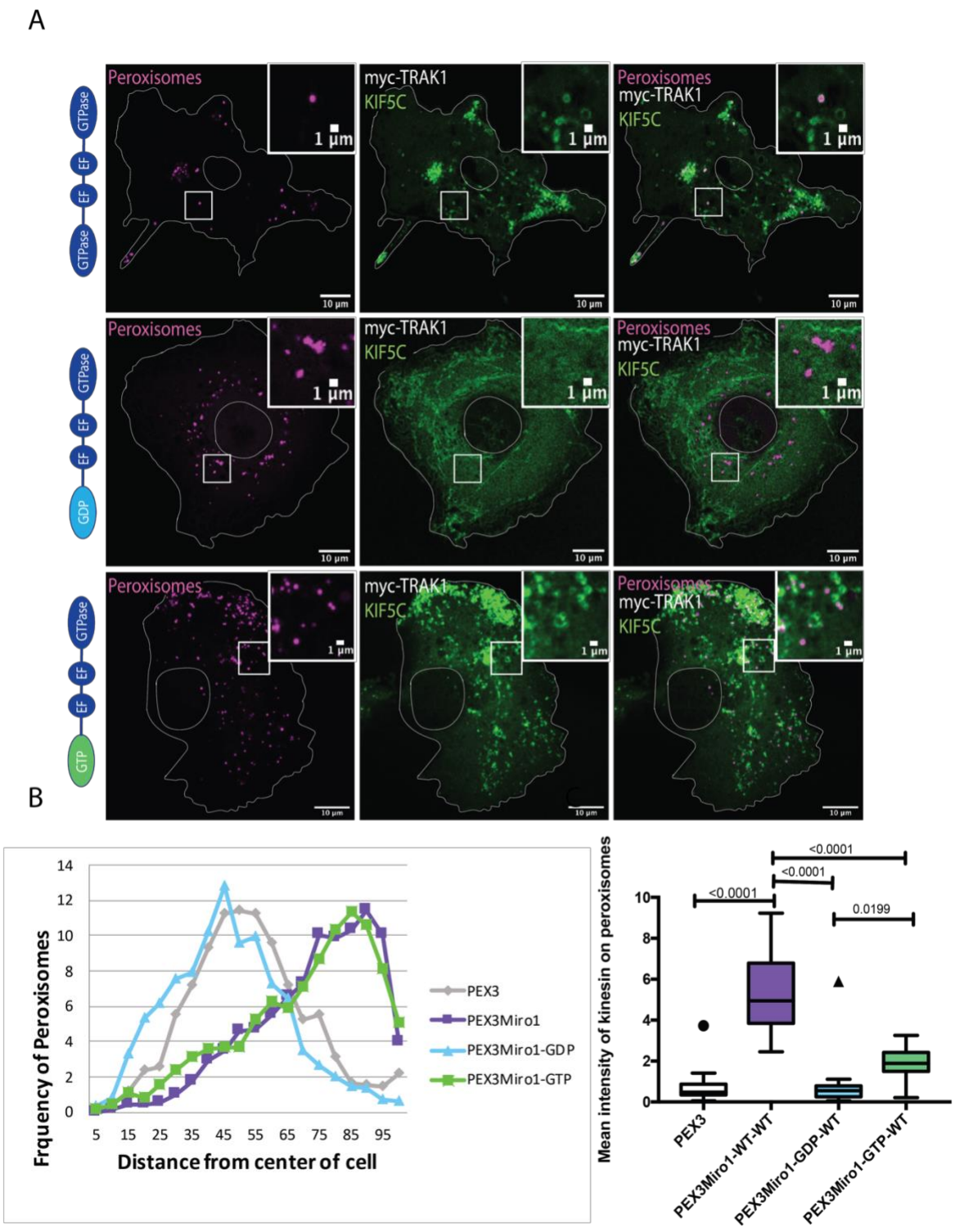


Figure 2.6 The nGTPase of Miro1 regulates co-localization with KIF5C

Figure 2.6 The nGTPase of Miro1 regulates co-localization with KIF5C cont.
Overexpression of PEX3Miro1 nGTPase mutants identifies a GTPase specific difference in Miro1 interaction with KIF5C

- *A) Overexpression of mTurquoise-SRL (marked as "peroxisomes" in magenta) with 6xhis-mRFP-PEX3Miro1 wild-type and nGTPase mutants (GDP-locked then GTP-locked) (mRFP signal not shown) with myc-TRAK1 and mCitrine-KIF5C in COS-7 cells which have been fixed 48 hours after transfection.*
- *B) Quantification of PEX3Miro1 peroxisome distance from the center of the cell using the DoveSonoPro macro. A comparison of the % frequency of peroxisomes in concentric shells radiating outwards from the center of the cell.*
- *C) Quantification of the co-localization of PEX3Miro1 wild-type and the PEX3Miro1 GTPase mutant peroxisomes with mCitrine-KIF5C, shown as the mean intensity of KIF5C signal on PEX3Miro1 peroxisomes. The quantification is represented as 'Box and Whiskers' plots. The line indicates the median. For demonstrating statistical significance, two-tailed unpaired t-test with Welch correction was used. Outliers are represented as single plot points and were included in all statistical calculations. N=15 cells over 3 biological replicates.*

Because the PEX3Miro1 constitutively inactive GDP mutant does not co-localize with KIF5C, we next looked to see whether the nGTPase of Miro1 also regulates the interaction between PEX3Miro1 and TRAK1. Mechanisms for the shedding of the KIF5C motor from the mitochondrial motor-adaptor complex have been reported but the dissociation of the TRAK adaptor from Miro1 rarely occurs (van Spronsen et al., 2013; Wang et al., 2011; Chung et al., 2016). Upon overexpression of mCitrine-TRAK1, we were surprised to find that the overexpressed TRAK1 adaptor fails to co-localize with peroxisomes that are positive for the PEX3Miro1 constitutively inactive GDP mutant (figure 2.7 A, B). We also tested the ability of the PEX3Miro1 constitutively inactive GDP mutant to co-localize with mCitrine-TRAK2. We found that the N-terminal GTPase regulates the co-localization of mCitrine-TRAK1 and mCitrine-TRAK2 with PEX3Miro1 N-terminal GDP mutant peroxisomes (figure 2.7C, D). In contrast, when the constitutively active GTP PEX3Miro1 mutant is localized to peroxisomes, both mCitrine-TRAK1 and mCitrine-TRAK2 are co-localized with peroxisomes. We quantified the amount of TRAK1 and TRAK2 co-localized to PEX3Mio1 peroxisomes using the previously described colocalization macro. These results were consistent with our previous observations that

the constitutively active GTP PEX3Miro1 mutant co-localizes with less KIF5C than wild-type PEX3Miro. This time, we found evidence that the constitutively active GTP PEX3Miro1 mutant co-localizes with less TRAK1 and TRAK2 than wild-type PEX3Miro1 (figure 2.7 B, D). Because the PEX3Miro1 nGTPase regulates interaction with TRAK2, we were not surprised to find that the co-localization of PEX3Miro1 peroxisomes with mCitrine-KIF5C was also regulated by the Miro1 nGTPase upon overexpression of myc-TRAK2 and mCitrine-KIF5C (figure 2.8).

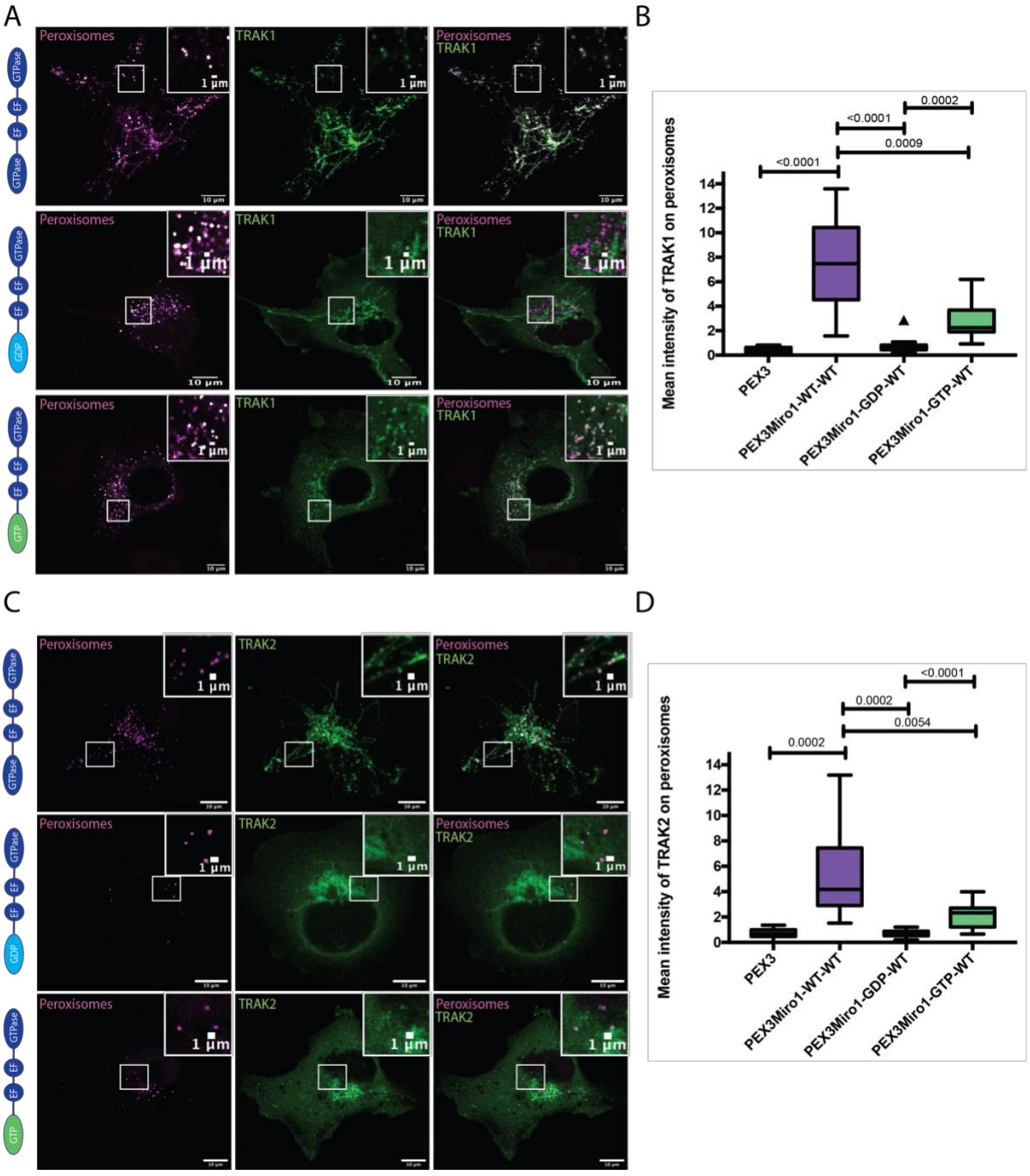


Figure 2.7 The PEX3Miro1 nGTPase regulates co-localization of TRAK1 and TRAK2 with PEX3Miro1.

Overexpression of PEX3Miro1 nGTPase mutants shows a GTPase dependent regulation of TRAK1 and TRAK2 co-localization with PEX3Miro1 peroxisomes.

A) Overexpression of mTurquoise-SRL (shown in magenta) with 6xhis-mRFP-PEX3Miro1 wild-type and nGTPase GDP and GTP mutants (mRFP not shown) with mCitrine-TRAK1 in COS-7 cells. Cells have been fixed 48 hours after transfection.

Figure 2.7 The PEX3Miro1 nGTPase regulates co-localization of TRAK1 and TRAK2 with PEX3Miro1 cont.

B) Quantification of the co-localization of PEX3Miro1 wild-type and the PEX3Miro1 GTPase mutant positive peroxisomes with mCitrine-TRAK1, shown as the mean intensity of TRAK1 signal on PEX3Miro1 peroxisomes. The quantification is represented as 'Box and Whiskers' plots. The line indicates the median. For demonstrating statistical significance, two-tailed unpaired t-test with Welch correction was used. Outliers are represented as single plot points and were included in all statistical calculations. N=15 cells over 3 biological replicates.

C) Overexpression of mTurquoise-SRL (shown in magenta) with 6xhis-mRFP-PEX3Miro wild-type and nGTPase GDP and GTP mutants (mRFP signal not shown) with mCitrine-TRAK2 in COS-7 cells. Cells are fixed 48 hours after transfection.

D) Quantification of the co-localization of PEX3Miro1 wild-type and the PEX3Miro1 GTPase mutant peroxisomes with mCitrine-TRAK2, shown as the mean intensity of TRAK2 signal on PEX3Miro1 peroxisomes. The quantification is represented as 'Box and Whiskers' plots. The line indicates the median. For demonstrating statistical significance, two-tailed unpaired t-test with Welch correction was used. Outliers are represented as single plot points and were included in all statistical calculations. N=15 cells over 3 biological replicates.

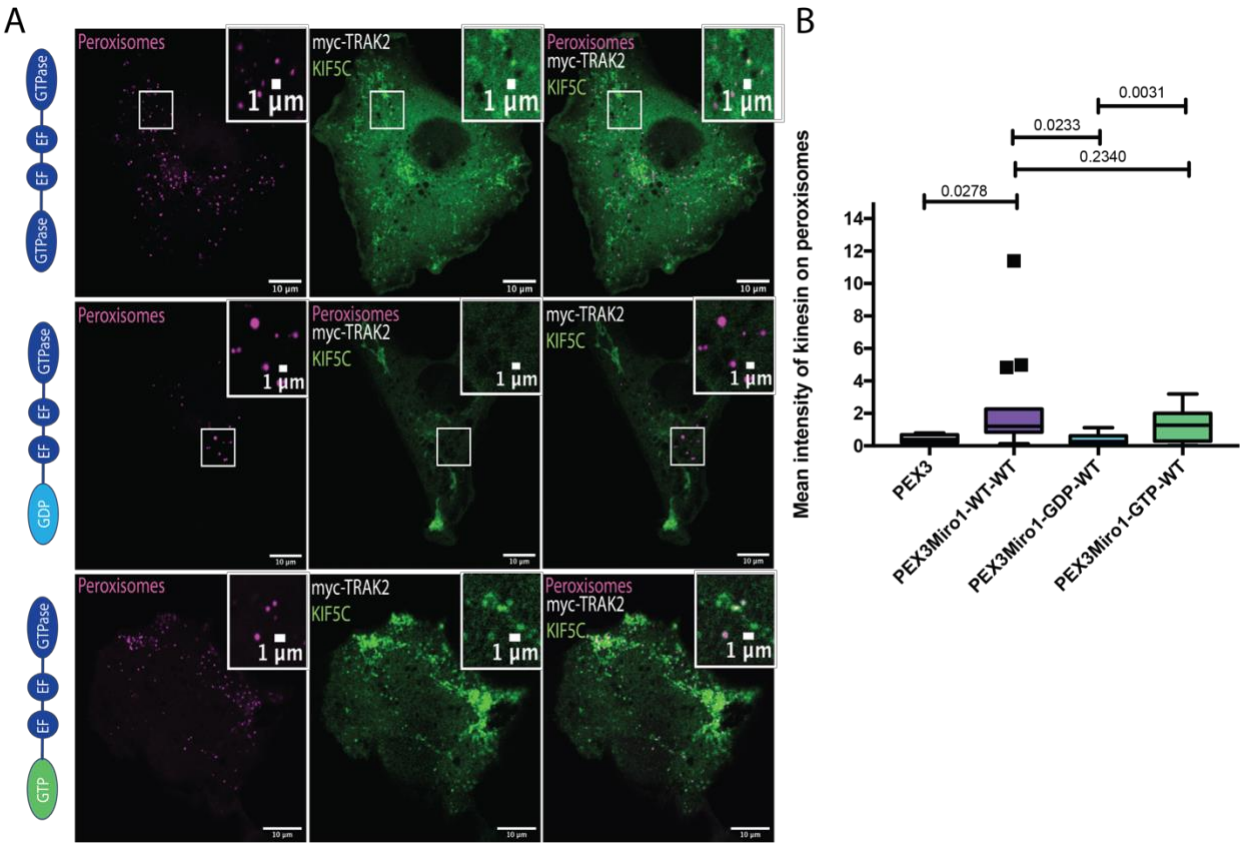


Figure 2.8 The PEX3Miro1 nGTPase regulates co-localization of KIF5C in the presence of TRAK2

Figure 2.8 The PEX3Miro1 nGTPase regulates co-localization of KIF5C in the presence of TRAK2 cont.

Overexpression of PEX3Miro1 nGTPase shows a GTPase dependent interaction with KIF5C dependent on the overexpression of TRAK2.

A) Overexpression of mTurquoise-SRL peroxisome marker (shown in magenta) with 6xhis-mRFP-PEX3Miro wild-type and GTPase mutants (mRFP signal not shown) with myc-TRAK2 (myc signal not shown) and mCitrine-KIF5C in COS-7 cells.

B) Quantification of the co-localization of PEX3Miro1 wild-type and the PEX3Miro1 GTPase mutant peroxisomes with mCitrine-KIF5C, shown as the mean intensity of mcitrine-KIF5C signal on PEX3Miro1 peroxisomes. The quantification is represented as 'Box and Whiskers' plots. The line indicates the median. For demonstrating statistical significance, two-tailed unpaired t-test with Welch correction was used. Outliers are represented as single plot points and were included in all statistical calculations. N=15 cells over 3 biological replicates.

All together we find that the N-terminal GTPase domain of PEX3Miro1 regulates the assembly of the motor-adaptor complex. The nGTPase constitutively inactive mutation results in a loss of co-localization between PEX3Miro1 peroxisomes with both the KIF5C motor and the TRAK1 and TRAK2 adaptor proteins. Interestingly we find that although PEX3Miro1 constitutively active nGTPase mutant peroxisomes do co-localize with KIF5C and the TRAK1 and TRAK2 adaptor proteins, it does not co-localize with the same quantity of KIF5C and TRAK1/2 as the peroxisomes which co-localize with the wild-type PEX3Miro1 construct. This difference in co-localization suggests that the nGTPase GTP locked mutation is not as efficient as wild-type PEX3Miro1 at co-localizing with TRAK and KIF5C, which may be due to slight differences in the protein conformation of the GTP locked mutant or the absence of a bound GTP to the active site. Given the functional difference that we were able to observe in the nGTPase mutants, we next utilized the PEX3Miro1 construct to explore the function of the Miro1 cGTPase domain.

Miro1 C-terminal GTPase regulation of the mitochondrial motor-adaptor complex

The cGTPase domain of Miro1 has previously been investigated but overexpression studies have failed to identify its function (Fransson et al., 2006). To study the cGTPase domain of Miro1, we

made single point mutations in the cGTPase of PEX3Miro1 which altered the GTPase domain to reflect either the constitutively active GTP mutation or the constitutively inactive GDP mutation. We co-overexpressed the mTurquoise-SRL peroxisome marker with these PEX3Miro1 cGTPase mutants in COS-7 cells with myc-TRAK1 and mCitrine-KIF5C and looked for differences in the PEX3Miro1 cGTPase mutant peroxisome's ability to co-localize with components of the motor-adaptor complex. Analysis of the co-localization of the PEX3Miro1 cGTPase mutant peroxisomes with myc-TRAK1 and mCitrine-KIF5C found that both mutants co-localized with KIF5C despite the GTPase mutations (figure 2.9 A). Quantification of the co-localization of the PEX3Miro1 mutant peroxisomes with KIF5C found a difference between wild-type PEX3Miro1 and the cGTPase constitutively inactive mutant peroxisomes co-localization with KIF5C. The constitutively inactive mutant peroxisomes co-localized with less KIF5C than wild-type PEX3Miro1 peroxisomes (figure 2.9B). We also found a difference between the co-localization of KIF5C with the PEX3Miro1 cGTPase constitutively active and constitutively inactive mutant peroxisomes (figure 2.9B). Interestingly, wild-type PEX3Miro1 peroxisomes co-localize with a similar amount of mCitrine-KIF5C as the cGTPase constitutively active mutant peroxisomes. This suggests that the cGTPase constitutively active mutant might be more similar to wild-type PEX3Miro1 than the nGTPase constitutively active mutant. Quantification of the distribution of peroxisomes from the center of the cell with co-overexpressed PEX3Miro1 or PEX3Miro1 cGTPase mutants with myc-TRAK1 and mCitrine-KIF5C did not find a difference between the distribution of PEX3Miro1 and PEX3Miro1 cGTPase constitutively active mutant peroxisomes but did find a small difference between PEX3Miro1 wild-type and the cGTPase constitutively inactive mutant peroxisomes (figure 2.9C).

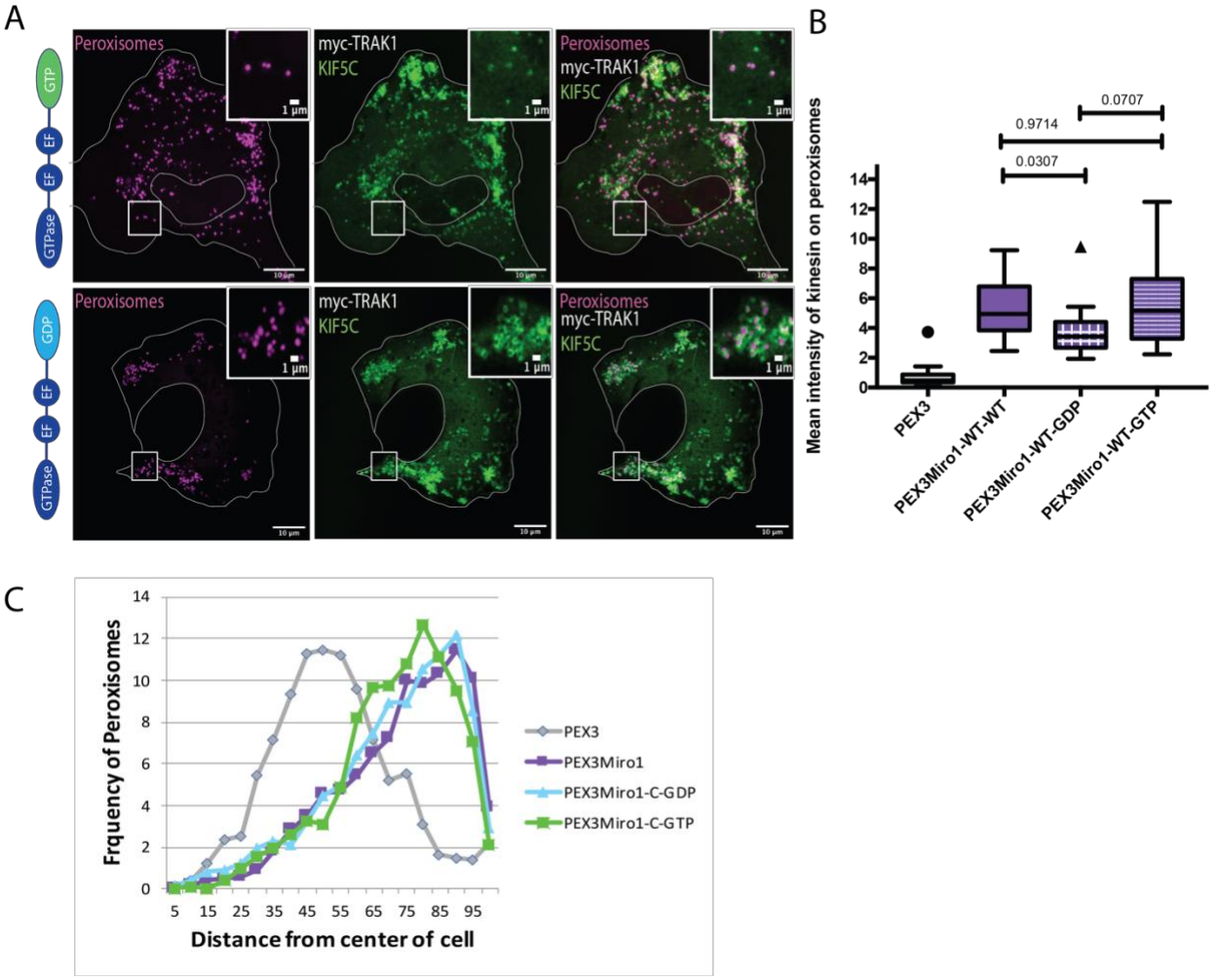


Figure 2.9 Miro1 C-terminal GTPase regulation of the mitochondrial motor-adaptor complex
Overexpression of Miro1 cGTPase mutants shows that PEX3Miro1 and PEX3Miro1 cGTPase mutants co-localize with KIF5C

- *A) Overexpression of mTurquoise-SRL peroxisome marker with PEX3Miro wild-type and cGTPase mutants (signal not shown) with myc-TRAK1 (signal not shown) and mCitrine-KIF5C in COS-7 cells. Cells are fixed 48 hours after transfection.*
- *B) Quantification of PEX3Miro1and PEX3Miro1 cGTPase mutant peroxisome distribution using the DoveSonoPro macro. A comparison of the % frequency of peroxisomes in concentric shells radiating outwards from the center of the cell.*
- *C) Quantification of the co-localization of PEX3Miro1 wild-type and the PEX3Miro1 cGTPase mutant peroxisomes with KIF5C in the presence of overexpressed myc-TRAK1, shown as the mean intensity of mCitrine-KIF5C signal on PEX3Miro1 peroxisomes. The quantification is represented as 'Box and Whiskers' plots. The line indicates the median. For demonstrating statistical significance, two-tailed unpaired t-test with Welch correction was used. Outliers are represented as single plot points and were included in all statistical calculations. N=15 cells over 3 biological replicates.*

Miro1 N-terminal GTPase is the dominant GTPase for regulation of motor-adaptor complex assembly

To further investigate a possible role for the PEX3Miro1 cGTPase in regulating the interaction between PEX3Miro1 and the motor-adaptor complex, we made PEX3Miro1 GTPase double mutants with either both the nGTPase and cGTPase in the constitutively active conformation or the nGTPase and cGTPase in the constitutively inactive conformation. When we overexpressed the double PEX3Miro1 GTPase mutants in COS-7 cells with the mTurquoise-SRL peroxisome marker, myc-TRAK1, and mCitrine-KIF5C, we found that the double mutant phenotype mimicked that of the nGTPase mutation. Peroxisomes with the PEX3Miro1 double constitutively inactive GTPase mutant did not co-localize with mCitrine-KIF5C but the those with wild-type or the double constitutively active GTPase mutant did (figure 2.10A). To further probe the interplay of the two GTPase domains, we also made double "on-off" or "off-on" mutants that have either locked the nGTPase in the constitutively inactive state and the cGTPase in the constitutively active or the opposite arrangement where the nGTPase has the constitutively active mutation and the cGTPase has the constitutively inactive mutation (figure 2.10 B). Quantification of the co-localization between the PEX3Miro1- positive peroxisomes and mCitrine-KIF5C found a significant difference between the wild-type PEX3Miro1 construct and the PEX3Miro1 double constitutively inactive mutant, as well as the double "off-on" mutant (nGTPase is constitutively inactive and the cGTPase is constitutively active) (figure 2.10C). Interestingly, we found that the "on-off" PEX3Miro mutant peroxisomes co-localized with less mCitrine-KIF5C than the double constitutively active mutant, suggesting that the cGTPase does play a role in enhancing the effect of the nGTPase on the interaction with KIF5C. In considering mCitrine-KIF5C recruitment to peroxisomes by each of the PEX3Miro1 GTPase mutants, the cGTPase constitutively active mutant is most similar to recruitment by wild-type PEX3Miro1(figure 2.10E). Together, these

results indicate that the PEX3Miro1 nGTPase is dominantly regulating the assembly of the motor-adaptor complex, but the cGTPase plays a role in this regulation of the interaction between PEX3Miro1 with TRAK1 and KIF5C. The double constitutively active GTPase mutant peroxisomes co-localize with more KIF5C than the nGTPase constitutively active mutant. This suggests that the PEX3Miro1 cGTPase could play a role in increasing the co-localization of KIF5C to the motor-adaptor complex but further experiments are needed to test this hypothesis. The distribution of PEX3Miro1 or PEX3Miro1 GTPase mutant peroxisomes are consistent with previous results, with the constitutively inactive nGTPase mutation causing peroxisome localization to be closer to the center of the cell (figure 2.10D). Quantification of the data for the distribution of peroxisomes for the “off-on” and “on-off” mutants is in process.

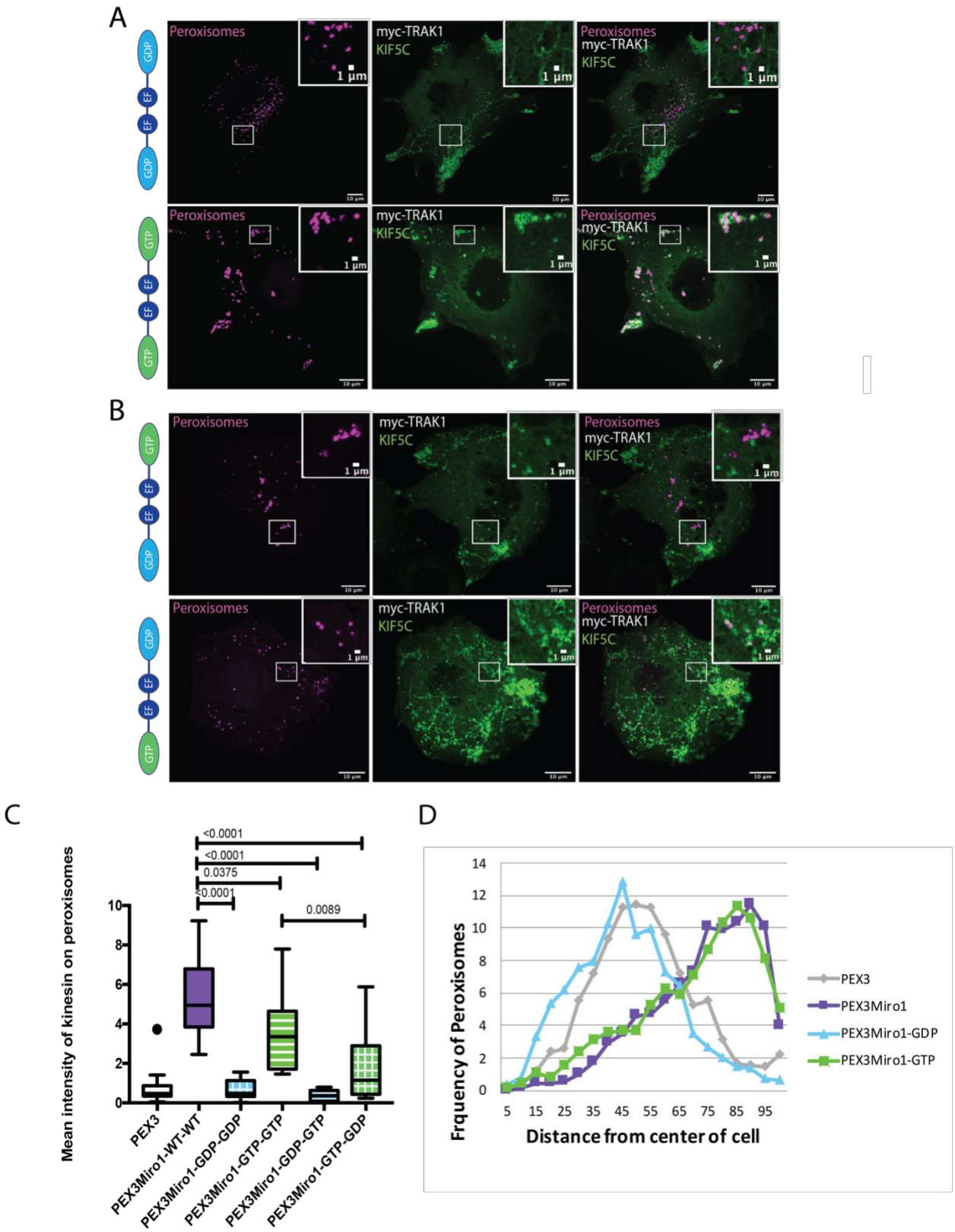


Figure 2.10 The nGTPase of Miro1 is the dominant GTPase regulator for co-localization with KIF5C

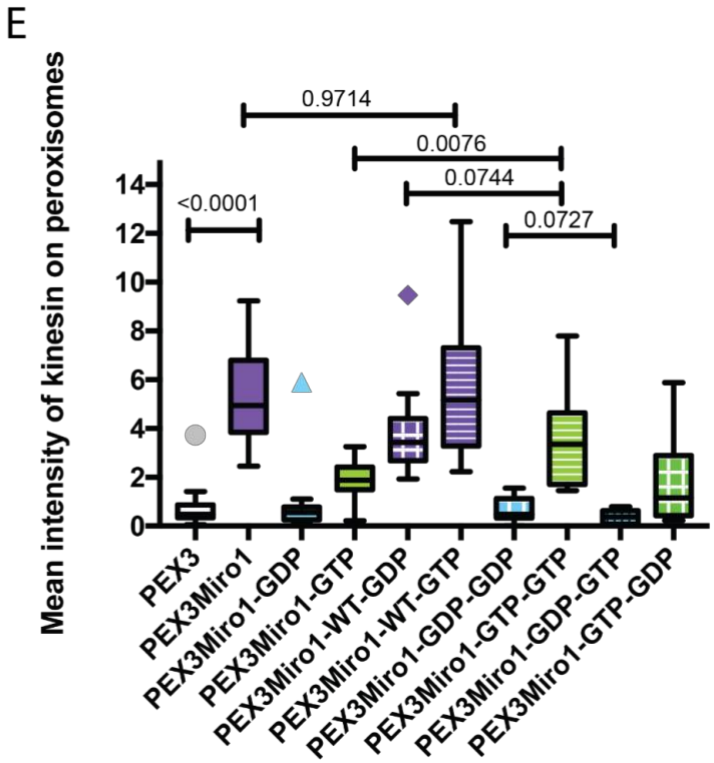


Figure 2.10 The nGTPase of Miro1 is the dominant GTPase regulator for co-localization with KIF5C cont.

Overexpression of PEX3Miro1 nGTPase and cGTPase double mutants with myc-TRAK1 and mCitrine-KIF5C shows an nGTPase dominant regulation of co-localization with KIF5C.

- *A) Overexpression of mTurquoise-SRL peroxisome marker (shown in magenta) with PEX3Miro1 nGTPase and cGTPase double mutants (signal not shown) with myc-TRAK1 (signal not shown) and mCitrine-KIF5C in COS-7 cells. Cells were fixed 48 hours after transfection.*
- *B) Overexpression of mTurquoise-SRL peroxisome marker (shown in magenta) with PEX3Miro1 nGTPase and cGTPase double "off-on" or "on-off" mutants (signal not shown) with myc-TRAK1 (signal not shown) and mCitrine-KIF5C in COS-7 cells. Cells were fixed 48 hours after transfection.*
- *C) Quantification of the co-localization of PEX3Miro1 wild-type and the PEX3Miro1 nGTPase and cGTPase double mutant peroxisomes with mCitrine-KIF5C, shown as the mean intensity of KIF5C signal on PEX3Miro1 peroxisomes. The quantification is represented as 'Box and Whiskers' plots. The line indicates the median. For demonstrating statistical significance, two-tailed unpaired t-test with Welch correction was used. Outliers are represented as single plot points and were included in all statistical calculations. N=15 cells over 3 biological replicates.*
- *D) Quantification of PEX3Miro1 nGTPase and cGTPase double mutant peroxisome distribution using the DoveSonoPro macro. A comparison of the % frequency of peroxisomes in concentric shells radiating outwards from the center of the cell.*
- *E) Quantification of all of the PEX3Miro1 GTPase mutant peroxisome's co-localization with mCitrine-KIF5C in the presence of overexpressed myc-TRAK1. Co-localization is shown as the mean intensity of KIF5C signal on PEX3Miro1 peroxisomes. The quantification is represented as 'Box and Whiskers' plots. The line indicates the median. For demonstrating statistical significance, two-tailed unpaired t-test with Welch correction was used. Outliers are represented as single plot points and were included in all statistical calculations. N=15 cells over 3 biological replicates.*

These cGTPase mutant experiments and double mutant experiments were also completed with mCitrine-TRAK1 and mCitrine-TRAK2. Quantification of the co-localization of mCitrine-TRAK1 or mCitrine-TRAK2 with the cGTPase PEX3Miro1 mutant peroxisomes and double PEX3Miro1 mutant peroxisomes found the same general trend in co-localization with PEX3Miro1 peroxisomes that was found with mCitrine-KIF5C. The nGTPase was the dominant GTPase for regulating interaction with the motor-adaptor complex. The nGTPase constitutively active double mutants co-localized with less TRAK1 and TRAK2 than wild-type PEX3Miro (figure 2.11 A,B). In summary, we find that the nGTPase of Miro1 is an important regulator of motor-adaptor complex assembly and that the cGTPase of Miro1 plays a subtler role in the regulation of the assembly of the motor-adaptor complex (figure 2.12).

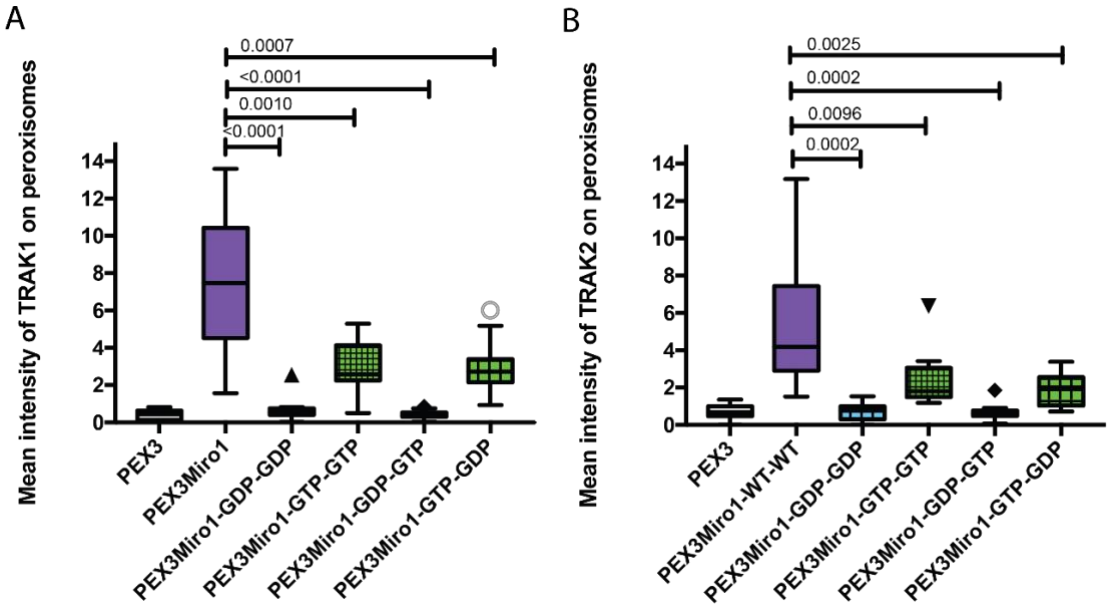
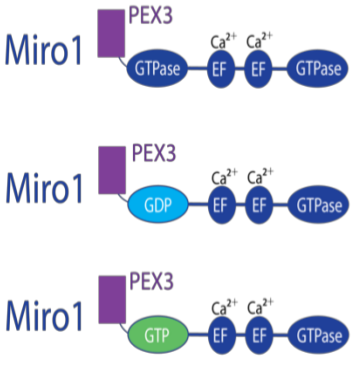


Figure 2.11 The nGTPase of Miro1 is the dominant GTPase regulator for co-localization with TRAK1 and TRAK2

A) Quantification of the co-localization of PEX3Miro1 wild-type and the PEX3Miro1 nGTPase and cGTPase double mutant peroxisomes with mCitrine-TRAK1, shown as the mean intensity of TRAK1/2 signal on PEX3Miro1 peroxisomes. The quantification is represented as 'Box and Whiskers' plots. The line indicates the median. For demonstrating statistical significance, two-tailed unpaired t-test with Welch correction was used. Outliers are represented as single plot points and were included in all statistical calculations. N=15 cells over 3 biological replicates.

B) Quantification of the co-localization of PEX3Miro1 wild-type and the PEX3Miro1 nGTPase and cGTPase double mutant peroxisomes with mCitrine-TRAK2.



N-term	C-term	TRAK1 + KIF5C	TRAK1	TRAK2	TRAK2 + KIF5C
WT	WT	✓	✓	✓	✓
	GDP	✓	✓	✓	✓
	GTP	✓	✓	✓	✓
GDP	WT	✗	✗	✗	✗
	GDP	✗	✗	✗	✗
	GTP	✗	✗	✗	✗
GTP	WT	✓	✓	✓	✓
	GDP	✓	✓	✓	✓
	GTP	✓	✓	✓	✓

Figure 2.12 Summary table of PEX3Miro1 nGTPase and cGTPase regulation with components of the motor-adaptor complex.
A summary of PEX3Miro1 and PEX3Miro1 nGTPase and cGTPase mutant's co-localization with TRAK1, TRAK2, and KIF5C.

PEX3Miro1 can drive nGTPase-dependent redistribution of peroxisomes in hippocampal neurons

Because PEX3Miro1 is able to change the distribution of peroxisomes in COS-7 cells when myc-TRAK1 and mCitrine-KIF5C are co-overexpressed, we hypothesized that this increased peroxisome distribution away from the center of the cell would also be evident in hippocampal rat neurons. Although there is a distinct distribution difference for PEX3Miro1 peroxisomes based on their nGTPase domain in COS-7 cells, it is not always easy to visualize plus-end and minus-end directed motility differences in COS-7 cells. To further explore nGTPase dependent PEX3Miro1 peroxisome distribution, we overexpressed the mTurquoise-SRL peroxisome marker with wild-type PEX3Miro1 and nGTPase PEX3Miro1 mutants in rat hippocampal neurons. Neurons were transfected on DIV5 and fixed on DIV7. We found that PEX3 control peroxisomes are mostly localized to the soma of hippocampal neurons, however; when we

overexpress wild-type PEX3Miro1 along with myc-TRAK1 and mCitrine-KIF5C, the peroxisomes almost entirely leave the soma and accumulate in neurites (figure 2.13). This re-distribution of PEX3Miro1 peroxisomes also occurs with the overexpression of the constitutively active nGTPase mutant. Alternatively, when the constitutively inactive nGTPase mutant is overexpressed with myc-TRAK1 and mCitrine-KIF5C, we found that the vast majority of the peroxisomes accumulated in the soma of the neuron and that very few peroxisomes were in the neurites (figure 2.13A). We have collected data to analyze peroxisome distribution in 15 cells each for PEX3 and PEX3Miro1 constructs over 3 biological replicates and are in the process of quantifying this difference. We plan to quantify this distribution by counting peroxisomes in the soma and neurites and representing the distribution of peroxisomes in either location as a fraction of the total number of peroxisomes in the neuron. We further plan to use hippocampal neurons to continue to study the difference in TRAK1 and TRAK2 dependent co-localization of KIF5C to PEX3Miro1 peroxisomes.

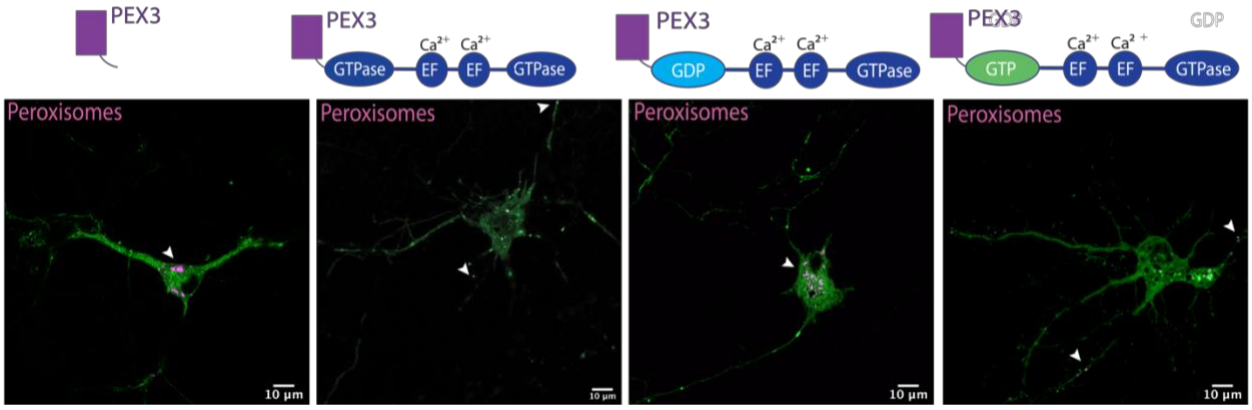


Figure 2.13 PEX3Miro1 dependent distribution of peroxisomes is regulated by the PEX3Miro1 nGTPase in hippocampal neurons
Overexpression of mTurquoise-SRL peroxisome marker (shown in magenta) with PEX3 control, wild-type PEX3Miro1, or PEX3Miro1 nGTPase mutants with myc-TRAK1 (signal not shown) and mCitrine-KIF5C (shown in green) in rat hippocampal neurons. Cells were fixed at DIV7. White arrows point to peroxisomes. PEX3Miro1 wild-type or nGTPase mutant status is represented by the cartoon above the image. Left to right: PEX3, PEX3Miro1, PEX3Miro1nGDP, PEX3Miro1nGTP.

PEX3Miro2 localization to peroxisomes does not co-localize with the motor-adaptor complex

Although Miro2 has long been believed to play a role in mitochondrial trafficking due to its similarities with Miro1, little evidence exists to support this notion. Because mitochondrial trafficking continues in Miro1 knockout models, it's been hypothesized that Miro2 can compensate for Miro1. In Miro1 knockout cells, overexpression of Miro2 can partially rescue mitochondrial trafficking. In contrast to the lethal effects of Miro1 knockout, Miro2 knockout studies have found that mice are viable and reported that Miro2 knockout does not affect mitochondrial localization or trafficking in neurons (López-Doménech et al., 2018). Interestingly, it's recently been reported that mitochondrial trafficking still occurs in Miro1/2 double knock out MEFs as well, suggesting that Miro2 may not be the protein responsible for mitochondrial trafficking in the absence of Miro1 (López-Doménech et al., 2018). To look for a functional difference in Miro1 and Miro2, we created a PEX3Miro2 construct that localizes to peroxisome membrane. The mislocalization of Miro2 to peroxisomes, allowed us to ask whether Miro2 interacts with the motor-adaptor complex in the absence of Miro1.

To answer this question, we co-overexpressed PEX3Miro2 with the mTurquoise-tagged peroxisome marker, myc-TRAK1, and mCitrine-KIF5C (figure 2.14A). We were surprised to find that PEX3Miro2 peroxisomes did not co-localize with mCitrine-KIF5C and did not have an effect on peroxisome distribution from the center of COS-7 cells. This result was unexpected, and we hypothesized that it was likely just the KIF5C motor that was not co-localizing with PEX3Miro2 peroxisomes. We further tested the ability of mCitrine-TRAK1 and mCitrine-TRAK2 to co-localize to PEX3Miro2 peroxisomes and were again amazed to find that neither TRAK adaptor protein was co-localized to peroxisomes (figure 2.15).

We hypothesized that although wild-type PEX3Miro2 does not co-localize with TRAK1, TRAK2, or KIF5C, there might be a GTPase-dependent regulation of this interaction, where PEX3Miro2 only co-localizes with the motor-adaptor complex as needed. Preliminary data that we have collected, and are in the process of repeating, has shown that none of the nGTPase or cGTPase PEX3Miro2 mutations alters PEX3Miro2 peroxisome's ability to co-localize with components of the motor-adaptor complex (data not shown). Although we are still collecting this data and quantifying the co-localization of PEX3Miro2 peroxisome with the components of the motor-adaptor complex, we have yet to observe any cell with even partial co-localization of the PEX3Miro2 construct with TRAK1/2 or KIF5C. Initial observations suggest that PEX3Miro2 does distribute PEX3Miro2 peroxisomes differently than PEX3Miro1 and this will be further studied using the DoveSonoPro distribution macro.

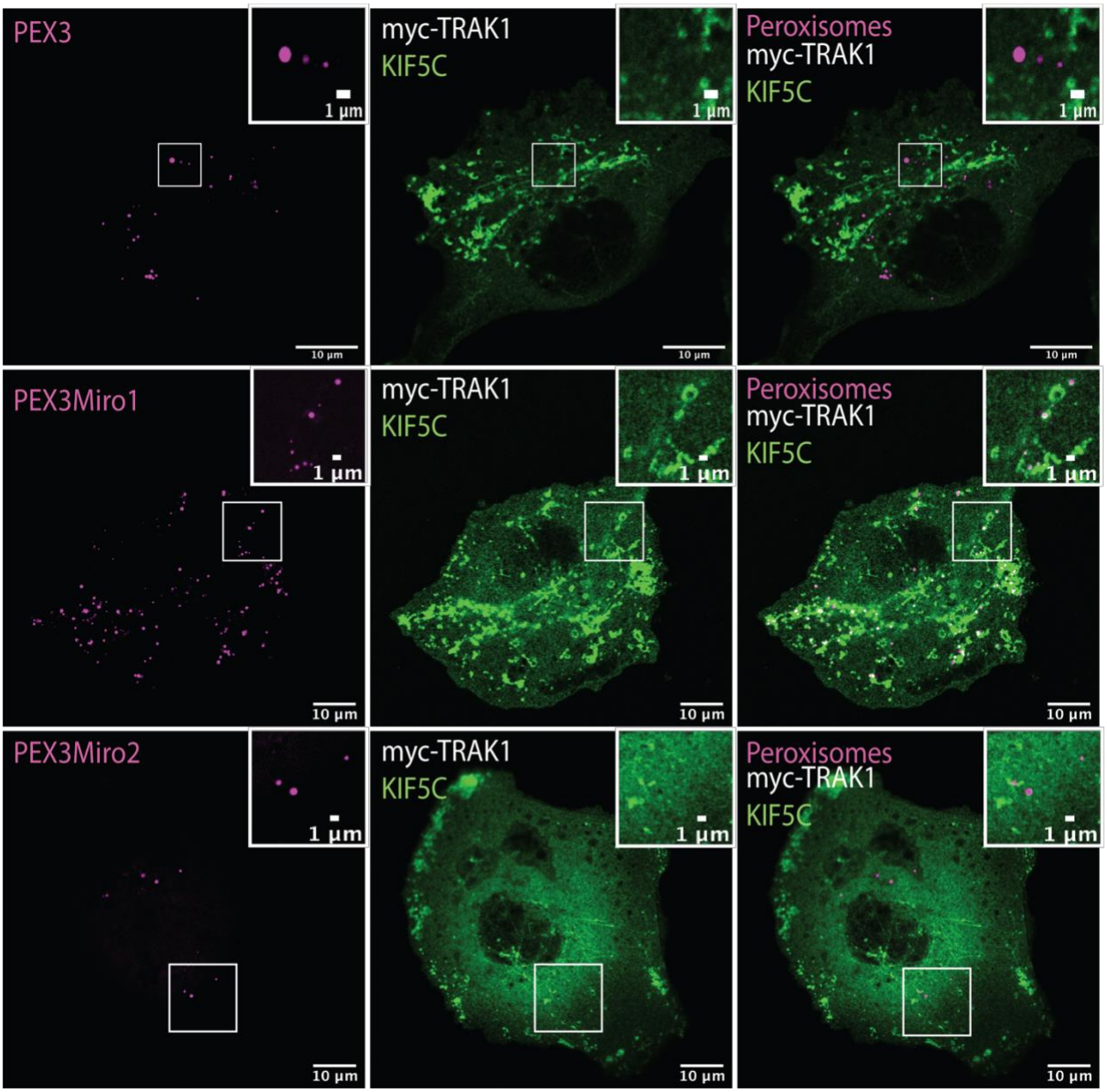


Figure 2.14 PEX3Miro2 localization to peroxisomes does not elicit mislocalization of KIF5C to peroxisomes

Overexpression of mTurquoise-SRL peroxisome marker (shown in magenta) with PEX3 control, PEX3Miro1, and PEX3Miro2 (signal not shown) with myc-TRAK1 (signal not shown), and mCitrine-KIF5C in COS-7 cells. PEX3 control peroxisomes do not co-localize with mCitrine-KIF5C but PEX3Miro1 peroxisomes do. PEX3Miro2 peroxisomes do not co-localize with mCitrine-KIF5C.

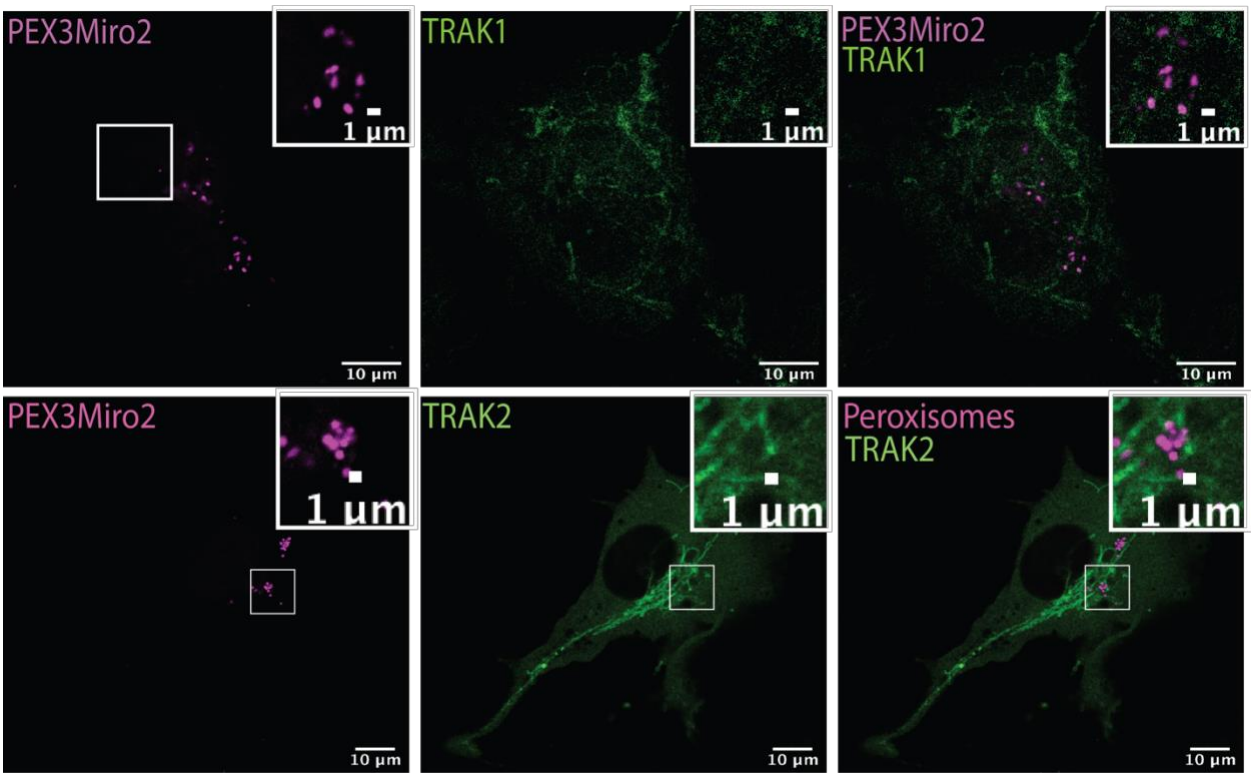


Figure 2.15 PEX3Miro2 localization to peroxisomes does not elicit mislocalization of TRAK1 or TRAK2 to peroxisomes
Overexpression of mTurquoise-SRL peroxisome marker (shown in magenta) with PEX3 control, PEX3Miro1, and PEX3Miro2 (signal not shown) with mCitrine-TRAK1 or mCitrine-TRAK2 in COS-7 cells. PEX3Miro2 peroxisomes do not co-localize with mCitrine-TRAK1 or mCitrine-TRAK2

Discussion

In this chapter, I describe a mechanism for Miro nGTPase specific regulation of the assembly of the motor-adaptor complex on PEX3Miro1 peroxisomes. We find that the PEX3Miro1 construct is sufficient for recruitment of the TRAK1/2 adaptor proteins to peroxisomes as well as sufficient for the recruitment of the kinesin-1 motor protein, KIF5C. The localization of the mitochondrial motor-adaptor complex to peroxisomes allows us to use PEX3Miro1 GTPase mutants to ask questions about the function of the Miro1 GTPase domains. We find that the Miro1 nGTPase domain regulates the ability of TRAK1/2 and KIF5C to localize to peroxisomes when the

nGTPase of Miro1 has a constitutively inactive GDP bound-like mutation. Further we identify a difference in TRAK1/2 interaction with KIF5C. We then use the PEX3Miro construct to ask questions about the functional difference between Miro1 and Miro2 and find that Miro2 is not sufficient for localization of the mitochondrial motor-adaptor complex to peroxisomes.

All together, we find that the PEX3Miro construct is a useful tool for asking questions about the mitochondrial motor-adaptor complex because it allows us to study the interaction of the proteins in the motor-adaptor complex without confounding our results with the presence of the endogenous motor-adaptor complex proteins. The PEX3Miro system also allows us to study how the motor-adaptor complex interacts without having to genetically alter the endogenous Miro proteins, which has been shown to decrease mitochondrial trafficking and negatively affect cell health (Nguyen et al., 2014; Kanfer et al., 2015; López-Doménech et al., 2016)

It has long been hypothesized that the nGTPase of Miro1 plays a role in regulating mitochondrial motility. The very first studies characterizing the Miro1 GTPase mutations found that Miro1 nGTPase mutants altered mitochondrial distribution in cells (Fransson et al., 2003). Since then, a number of studies have attempted to characterize the function of the Miro1 GTPase domains. These studies have found that the nGTPase constitutively inactive GDP mutation disrupts mitochondrial trafficking (Fransson et al., 2003; Fransson et al., 2006; MacAskill et al., 2008**)**. Studies have also found that the constitutively inactive nGTPase mutation leads to the dissociation of proteins that interact with Miro1, like Myo19 (Bocanegra et al., 2019). Studying how the Miro1 nGTPase regulates interaction with the motor-adaptor complex has been complicated. In MEF and U2Os Miro1 knockout models, mitochondria are still somewhat motile, and the TRAK adaptor proteins have been shown to still localize to mitochondrial

membrane making it impossible to tell whether the overexpression of a Miro1 nGTPase mutant regulates interaction of Miro1 with the motor-adaptor TRAK1/2 (López-Doménech et al., 2016). In this thesis, we have taken a novel approach for studying the function of the Miro1 GTPase domains and found an underlying mechanism for the mitochondrial distribution and motility phenotypes which have been known for years. The Miro1 nGTPase regulates the assembly of the motor-adaptor complex, including TRAK1, TRAK2, and KIF5C. This loss of interaction with the motor-adaptor complex explains why Miro1 nGTPase overexpression studies have seen a collapse of the mitochondrial network and a decreased mitochondrial motility phenotype when they overexpress the Miro1 nGTPase constitutively inactive mutant. We also find the Miro1 cGTPase plays a role in the assembly of the motor-adaptor complex and can increase the co-localization of PEX3Miro1 peroxisomes with KIF5C. This is an exciting finding as previous studies have not been able to identify a function of the Miro cGTPase domain. Further studies will be needed to fully understand how the two Miro1 GTPase domains coordinate the assembly of the motor-adaptor complex.

Using the PEX3Miro1 system, we also identified a difference in mCitrineTRAK1 and mCitrine-TRAK2 ability to co-localize with mTurquoise-KIF5C. Previous studies have reported a functional difference in the two TRAK homologues with, TRAK1 seeming to be the primary adaptor for KIF5C driven plus-end directed trafficking and TRAK2 seeming to be the primary adaptor for dynein driven minus-end directed trafficking (Brickley et al., 2005; MacAskill et al., 2008). Further studies are needed to fully elucidate the functional difference between TRAK1 and TRAK2 and we plan to continue utilizing the PEX3Miro system to study this difference, especially in neurons where plus-end and minus-end directed trafficking is more apparent.

One important factor missing in this thesis is how the dynein-dynactin motor is regulated by Miro1 GTPase domains. Studying the function of dynein using the PEX3Miro1 system has proven to be difficult with the components of the dynein-dynactin complex having either a cytosolic or microtubule localization upon overexpression, making it difficult to distinguish peroxisome specific recruitment of the motor from microtubule localization of the motor. To get around this, we have begun to utilize a truncation mutant of the dynein-dynactin complex protein, P150-glued that is not able to bind to microtubules, called P135. With overexpression of a fluorescently tagged P135, we are able to visualize the co-localization of the protein to PEX3Miro1 peroxisomes and think that this will be an exciting area of future study to further understand how the Miro1 GTPase domains regulates the motor-adaptor complex. We also think that the successful recruitment of P135 to PEX3Miro1 peroxisomes will prove to be a useful system for further studying the functional differences between TRAK1 and TRAK2. In addition to studying how PEX3Miro1 nGTPase regulates interaction with dynein, it will be interesting to also investigate how Miro1 nGTPase regulates the ability of kinesin-1 motors KIF5A and KIF5B to recruit to PEX3Miro1 peroxisomes. KIF5A and KIF5C are neuronally expressed. KIF5B is ubiquitously expressed across cell types. It will be interesting to use antibodies to look for the possible recruitment of endogenous KIF5B to PEX3Miro1 peroxisomes in COS-7 cells.

Miro2 has long been assumed to play a role in mitochondrial trafficking, with the overexpression of Miro2 able to partially compensate for a loss of Miro1 trafficking defect in Miro knockout models (López-Doménech; Norkett et al., 2016). The finding that PEX3Miro2 does not co-localize with components of the motor-adaptor complex was surprising and further complicates the understanding of the role of Miro2 in mitochondrial trafficking. The PEX3Miro construct will be a helpful tool as we continue to consider the function of Miro2 and its role in the

regulation of mitochondrial trafficking. We also look forward to further study of the Miro2 GTPase domains and the structure of Miro2 or any study which shows that the Miro2 GTPase domains are active.

For this thesis almost all of the data was collected using co-localization analysis. Although co-localization analysis can be used to tell whether two or more proteins are localized to similar places in a cell, co-localization does not necessarily mean that two proteins interact. Our model for those interactions is based on the extensive protein chemistry and immunoprecipitation studies that have been performed with the endogenous complex and overexpressed wildtype proteins and which we have confirmed. We have attempted immunoprecipitation of PEX3, PEX3Miro1, or PEX3Miro1 mutants to look changes in the co-immunoprecipitation of TRAK1 and KIF5C. PEX3Miro1 is able to co-immunoprecipitate TRAK1 and KIF5C but we were not able to see a PEX3Miro1 mutant specific difference in the ability to co-immunoprecipitate TRAK1 and KIF5C. These experiments were inconclusive probably because those precipitations necessarily bring down all the PEX3Miro1 in the cell, including that present on mitochondria (data shown and discussed in chapter 4). Our imaging studies, however, could exclusively quantify the complexes forming on peroxisomes in the absence of detectable endogenous Miro. Future directions for studying these interactions will be dependent on the purification of Miro1 and Miro1 GTPase mutants for in vitro biochemical characterization.

One important area of study for further understanding the role of the Miro GTPase domains will be the consideration of GEF and GAP proteins in the ability to regulate the Miro1 GTPase domains and thus the assembly of the motor-adaptor complex. Suzuki et al., 2014 have reported convincing evidence for the role of the Vibrio cholerae T3SS effector, VopE, as a GAP protein which disrupts a collapsed mitochondrial network phenotype upon overexpression. They further

show that VopE has the ability to regulate the GTPase activity of the Miro1 nGTPase domain. Their model suggests that VopE disrupts mitochondrial association with the mitochondrial network in an attempt to disrupt typical cellular signaling that would initiate the innate immune response upon infection with Vibrio cholerae. This GAP dependent disruption of the mitochondrial network fits nicely with our model where the Miro1 nGTPase domain regulates the assembly of the mitochondrial motor adaptor complex, disrupting Miro1's ability to interact with the proteins that connect it to microtubules. The further study and identification of endogenous GAP proteins, which have the ability to regulate Miro1, will be an exciting area of research.

Altogether, we propose a model for Miro GTPase regulation of the mitochondrial motor-adaptor complex where the GTP-bound state of the Miro1 nGTPase facilitates the recruitment of the motor-adaptor complex components TRAK1/2 and kinesin. When the Miro1 nGTPase undergoes hydrolysis the bound GTP is hydrolyzed to GDP which leads to a dissociation of the components of the motor-adaptor complex from Miro1 and leads to the disruption of mitochondrial trafficking.

Experimental Procedures

Plasmids and Constructs: The following previously published DNA constructs were used in this study: pmTurquoise2-Perox (a gift from Dorus Gadella, Addgene plasmid # 36203 (Goedhart et al., 2012)), Myc-TRAK1, (Pekkurnaz et al., 2014), mCitrineKIF5C (Chung et al., 2016), PEX3 and PEX3Miro1 (Basu et al., 2020), mito-GFP (Chung et al., 2016), mCitrine-KIF5C (Addgene plasmid #61667), mycMiro1-V13, V427, N18, N432 were kindly provided by Dr. P. Aspenstrom (Karolinska Institute)

To make the myc-TRAK2 construct, the Myc-TRAK1 construct described in (Pekkurnaz et al., 2014) was linearized by PCR with the primers *KP51* and *KP52.* These primers were designed to omit the TRAK1 in the backbone. The TRAK2 cassette was amplified from the TRAK2 cDNA clone BC048093 from Transomic using KP48 and KP49 primer that had appropriate overhangs to the myc- vector. The linearized myc- vector was re-circularized after incorporating the TRAK2 cassette at the N-terminus of myc-TRAK2 by Gibson assembly. This construct was used to overexpress TRAK2 in all kinesin colocalization experiments.

To make the mCitrine-TRAK1 and mCitrineTRAK2 construct, the mCitrine-KIF5C construct described in Chung et al., 2016 was linearized by PCR with the primers *KP69* and *KP70.* These primers were designed to omit the KIF5C in the backbone. The TRAK1 and TRAK2 cassettes were amplified from the mycTRAK1 construct (Pekkurnaz et al., 2014) and myc-TRAK2 construct described in this paper using KP71 and KP72 primers and X2 and Y2 primers that had appropriate overhangs to the mCitrine- vector. The linearized mCitrine- vector was re-circularized after incorporating the TRAK1 and the TRAK2 cassette at the N-terminus of mCitrine-TRAK1 and mCitrine-TRAK2 by Gibson assembly. These constructs were used to

overexpress all TRAK1 and TRAK2 in all TRAK1/2 colocalization experiments.

The PEX3Miro1 construct was made by amplifying the first 592 amino acid residues from isoform 3 of human Miro1 as cloned in (Fransson et al., 2003) with the primers *pex-miro1_ForPrimer* and *pex-miro1_RevPrimer*. The pex3 peroxisomal targeting domain (amino acids 1-42) along with mRFP (i.e. Pex3-mRFP) was obtained by excising the FKBP from the pex3-mRFP-FKBP construct (a gift from Casper Hoogenraad (Kapitein et al., 2010)) using EcoR1 and AscI. This was followed by fusing the Miro1 PCR product into the Pex3-mRFP backbone by Gibson assembly.

PEX3Miro1_ForPrimer:

5'GAGGGCCGCCACTCCACCGGCGCCGGCGCGCCTCGCTCTAGATCCGGACTCAGAT CTCGA 3'

PEX3Miro1_RevPrimer:

5'GTGGTATGGCTGATTATGATCAATGAATTCTTAATAACTAGTAGATCCGGTGGATC CCGG 3'

PEX3Miro2 cloning strategy:

To generate the PEX3Miro2 construct, the first 593 amino acid residues were amplified from the Miro2 isoform isolated in (Fransson et al., 2003) with the primers *pex-miro2_ForPrimer* and *pex-miro2_RevPrimer.* A strategy similar to that used to make the PEX3Miro1 construct was then employed to build pex-miro2. The Miro2 amplicon (containing the first 593 amino acid residues) was assembled just downstream of the pex3-mRFP cassette in the linearized Pex3-mRFP contruct, (using EcoR1 and AscI) by Gibson Assembly.

All the mutants were made by Site directed Mutagenesis carried out on the PEX3Miro1 and PEX3Miro2 constructs

The nGTPase mutants were made using KP24, KP25, KP26, KP27.

Table 2.1 Primer sequences used in this study

Primer Name	Sequence	Note
KPrimer 24	GAAGAACTCACTGATTATGTC	forward primer T18N
KPrimer 25	CCAACTCTAGGTTCTCCCACC	reverse primer T18N
KPrimer 26	GAAGTTAGAGTTGGGAAGACA	forward primer P13V
KPrimer 27	TCCCACCAGCAGGATCCGCAC	reverse primer P13V
KPrimer 51	AGGAGGACTGAGTCTCGAGGGGGGGCC	forward primer to amplify myc-trak backbone with Trak2 overhangs
KPrimer 52	TTGACTCATTGGATCCGCCCG	Reverse primer to amplify myc-Trak backbone with Trak2 overhangs
KPrimer 69	GAGCTGTACAAGTCCGGAGCAGGAATGGC G	KIF5C Forward
KPrimer 70	TCCGGTGGATCCCTTCTGGTAGTGAGTGGA	KIF5C insert reverse
KPrimer 71	CACTACCAGAAGGGATCCACCGGATCTAGA	mTurquoise bb forward
KPrimer 72	TCCTGCTCCGGACTTGTACAGCTCGTCCAT	mTurquoise bb reverse
KPrimer 81	GCCCACTCCACAGGCCCCTAC	S430N reverse primer Miro2
KPrimer 102	AAGAACGCCTTCCTGCAGGCC	S430N forward primer Miro2
KPrimer 65	CTGAAGGAGGACGGAGCAGGTACTGGAGC A	mcit TRAK2 bb forward
KPrimer 66	CTGGGATTGACTTCCTGCTCCGGACTTGTA	mcitTRAK2 bb reverse
KPrimer 67	TCCGGAGCAGGAAGTCAATCCCAGAATGCA	mcit TRAK2 insert forward
KPrimer 68	AGTACCTGCTCCGTCCTCCTTCAGGACACC	mcit TRAK2 insert reverse
KPrimer 48	CGGGCGGATCCAATGAGTCAATCCCAGAAT GCA	forward primer to ampify Trak2 with Myc bb overhangs
KPrimer 50	CCCCCTCGAGACTCAGTCCTCCTTCAGGAC ACC	reverse primer to amplify TRAK2 with myc trak overhangs
KPrimer 51	AGGAGGACTGAGTCTCGAGGGGGGGCC	forward primer to amplify myc-trak bb with Trak2 overhangs
KPrimer 52	TTGACTCATTGGATCCGCCCG	Reverse primer to amplify myc-Trak bb with Trak2 overhangs
KPrimer 61	AACTAGCTTACGGGGAGCAGGTACTGGAGC	Mcit TRAK1 bb forward
KPrimer 62	AAAAACCAATGCTCCTGCTCCGGACTTGTA	Mcit Trak1 bb reverse
KPrimer 63	TCCGGAGCAGGAGCATTGGTTTTTCAATTC	mcit Trak1 insert forward
KPrimer 64	TCCAGTACCTGCTCCCCGTAAGCTAGTTTG	mcit TRAK1 insert reverse

Antibodies used for western blotting: For Western blots, the following primary antibodies were used at the stated dilutions: anti-human TRAK1 at 1:2000 (HPA005853, Sigma-Aldrich), anti-myc at 1:5000 (05- 724, EMD Millipore), anti-RFP at 1:1000 (SAB2702214, Sigma-Aldrich) and anti-6X-HIS at 1:1000 (MA1-21315, Thermo Fisher Scientific). For fluorescent detection (used for all quantitative blots) 680RD Donkey anti-Rabbit, 800CW Donkey anti-Mouse were used at 1:5000 (LiCor Biosciences) and all blots were scanned by the Odyssey® CLx Imaging System.

Cell Cultures and transfections: HEK293T and COS-7 cells were cultured in DMEM supplemented with L-glutamine, penicillin/streptomycin (Life Technologies), and 10% FBS (Atlanta Premium). Plasmid DNA transfections in HEK293T cells were performed with the calcium phosphate (Kingston et al., 2003). Plasmid DNA transfections in COS-7 cells were performed with PolyJet DNA (Signa Gen Laboratories) using the manufacturers guidelines. These cell lines were generally transfected 18-24 hours after plating and fixed 2 days later.

Hippocampal neurons were dissected and dissociated from E18 rat (Charles River) embryos as previously described ((Nie and Sahin, 2012)) and plated at 5–7 × 10^4/cm^2 density on glass bottom dishes (D35-20-1.5-N, Cellvis) coated with 20 μg/ml poly-L-Lysine (Sigma) and 4 μg/ml laminin (Life Technologies) and maintained in neurobasal (NB) medium supplemented with B27 (Life Technologies), L-glutamine, and penicillin/streptomycin, unless specified otherwise. Hippocampal neurons were transfected on DIV5 using Lipofectamine2000 (11668-019, Life Technologies) and imaged 2–3 days later.

Immunoprecipitations: For all immunoprecipitations, HEK293T cells were plated at 5.5x10^5 cells/well density in a 6-well plate and transfected the next day. Two days after transfection, cells

were washed once with ice- cold phosphate-buffered saline (PBS) and lysed in 600µl Pierce™ IP Lysis Buffer (Thermo Fisher, Catalog number: 87787) and protease inhibitor cocktail set III (539134-1SET, EMD Millipore) at 1:500 dilution. Lysates were centrifuged 10 min at 13,000 x g at 4°C and the clarified supernatants were collected. For immunoprecipitations of PEX3-6xhis-mRFP-Miro, anti-6X-HIS antibody were incubated for 1 hr at 4°C with 500 µl of the clarified supernatants of whole-cell lysates and then for 1 more hour with Dynabeads Protein G beads for immunoprecipitation (Thermo Fisher, catalog number: 10003D). The beads were pre-blocked in .2% TBST and washed three times with lysis buffer. After incubation beads were washed 5 times in lysis buffer and resuspended in 2xLaemmli buffer. 50% of this fraction was then separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were preblocked with 3% bovine serum albumin (w/v) in Tris-buffered saline with 0.1% Tween20 (TBST), followed by overnight incubation at 4C with primary antibodies in the blocking buffer. The blot was then washed 3 times with TBST and blotted with secondary antibodies for 1 hour at room temperature, before ECL imaging or being scanned by the Odyssey® CLx Imaging System.

Imaging Acquisition and Quantification: For imaging co-localization of TRAK1, TRAK2, and KIF5C on PEX3Miro1 peroxisomes, COS-7 cells were grown on glass bottom dishes (FD35-100, World Precision Instruments, Sarasota County, FL, United States of America). Cells were fixed and imaged at room temperature. Images were acquired using z-stacks with .3um slices ranging from the top to bottom of the cell. For imaging distribution of PEX3Miro1 peroxisomes in rat hippocampal neuron cultures were grown on glass bottom dishes (FD35-100, World Precision Instruments, Sarasota County, FL, United States of America). Neurons were fixed 48 hours after transfection and imaged at room temperature. Images were acquired using z-stacks

with .3um slices from the top to bottom of the neuron soma. Cells were imaged on a Leica SP8 laser scanning confocal microscope. Images were acquired using a 60X objective. A White Light Laser and Argon laser were used at 70% laser intensity and line scanning with 3 scans per line were used to acquire all images. The percentage of laser intensity used for the 458 spectra to capture mTurquoise markers was used at 5% throughout all experiments. The percentage of laser intensity used for the 515 spectra to capture mCitrine tagged constructs was used at 10% for all images. The percentage of laser intensity used for the 554 specra to capture all mRFP tagged constructs was used at 10% for all images. Gain was set at 100 for imaging mCitrine-TRAK1, mCitrine-TRAK2, mCitrine-KIF5C, mRFP-PEX3, and mRFP-PEX3Miro for all COS-7 and hippocampal neuron images. Only cells with visible PEX3 or PEX3Miro1 signal on peroxisomes at 100 gain with these laser conditions were imaged to control for differences in expression.

Quantification of peroxisome distribution was done as described in Basu et al., 2020 using the DoveSonoPro software (https://github.com/ThomasSchwarzLab). DoveSonoPro software allows for the cell outline and cell center to be selected by the user. Image file names were blinded for quantification and data was acquired and recorded by Ethan Shurberg.

Colocalization Quantification: Fixed Cos-7 cells expressing the SRL peroxisomal marker and PEX3 or PEX3Miro constructs. Cell masks were drawn in the peroxisome marker channel for each z-slice by tracing the perimeter of the cell and setting a threshold that excludes any non-peroxisomal signal.

Calculations:

> Total cell area without peroxisomes = total cell area (column2) - total peroxisomal area (column7) ---> Value 1

> Total kinesin intensity = total cell area (column2) * mean kinesin intensity (column4) ---> Value 2

> Total kinesin intensity outside peroxisomes = Total kinesin intensity (value 2) - total kinesin intensity on peroxisomes

> Mean kinesin intensity outside of peroxisomes = Total kinesin intensity outside peroxisomes / total cell area without peroxisomes (value 1)

> kinesin enrichment (column 8) = (mean kinesin intensity on peroxisomes - mean kinesin intensity outside peroxisomes)/mean kinesin intensity (column4)

Note: this value should come out to be less than 0 for de-enrichment and more than 0 for enrichment

> kinesin enrichment pex miro normalized (column 9) = kinesin enrichment/total pex-miro on peroxisomes (column 6)

> kinesin enrichment pex miro normalized and gain normalized (column 10) = (kinesin enrichment/kinesin gain) / (total pex-miro on peroxisomes/pex miro gain) (column 6)

Statistical Analysis: Statistical analysis was performed with GraphPad Prism v7.0. A two-tailed unpaired t-test with Welch correction was used to determine significances or differences between

populations in all co-localization experiment quantifications. All p-values were stated in the figures or figure legends.

Chapter 3.
Using PEX3Miro1 for further studies of mitochondrial dynamics.

Summary

In this Chapter, I discuss the use of the PEX3Miro1 system for further studying how proteins interact with the mitochondrial motor-adaptor complex. We've previously established that PEX3Miro1 is sufficient for the mislocalization of components of the motor-adaptor complex to peroxisomes and show that this recruitment of the motor-adaptor complex permits dissection of Miro1 function. Here, we use the strategy to further explore two areas of interest in the mitochondrial trafficking field. 1) Whether the syntaphilin (SNPH) anchor is necessary for Ca^{2+}-dependent regulation of mitochondrial trafficking and whether interaction with the motor-adaptor complex is sufficient for SNPH to anchor organelles. 2) How Miro1 interacts with the mitofusin protein Mfn2, and how this interaction between Miro1 and Mfn2 might regulate mitochondrial trafficking. We find that Ca^{2+}-dependent regulation of mitochondrial trafficking seems to occur without SNPH. We also establish that PEX3Miro1expression and PEX3Miro1-dependent recruitment of TRAK1 does not accomplish the recruitment of SNPH to peroxisomes. In studying the interaction between Mfn2 and PEX3Miro1, we find that PEX3Miro1 expression can cause the localization of Mfn2 to peroxisomes and that this interaction increases with the overexpression of TRAK1. However; we find that overexpression of a fluorescently tagged Mfn2 protein can also localize to peroxisomes with only the PEX3 control construct. This finding complicates our understanding of Mfn2/Miro1 interactions. These two studies are very preliminary but offer interesting results that warrant further study of Miro1 and SNPH interactions and of Miro1 and Mfn2 interactions.

Syntaphilin is not required for Ca^{2+} to arrest motors on the motor-adaptor complex.

A number of proteins outside of the mitochondrial motor-adaptor complex have been shown to interact with Miro1. These proteins link Miro1 to mitochondrial functions other than facilitating mitochondrial trafficking, like the anchoring of mitochondria and mitochondrial fission and fusion. The PEX3Miro1 system is a useful tool for studying how proteins interact with Miro1 and the other key components of the mitochondrial motor-adaptor complex.

Miro1 regulates mitochondrial motility through the Ca^{2+}-dependent regulation of the two pairs of Miro1 EF-hands. Wang et al., 2009 found that increased concentrations of intracellular calcium disrupt Miro1-dependent mitochondrial trafficking. This disruption in mitochondrial trafficking is due to the kinesin motor disassociating from microtubules while remaining attached to the mitochondrion via TRAK and Miro. Building on this model, another study reported that the anchor protein SNPH is necessary for this disruption in mitochondrial trafficking to occur (Chen and Sheng, 2009). SNPH is an anchor protein which has been shown to disrupt mitochondrial trafficking in axons by directly interacting with the KIF5 motor protein to inhibit kinesin motor-stepping (Cheng and Sheng, 2009). This model, however, is controversial because there is doubt that SNPH is present in dendrites and in non-neuronal cells where Ca^{2+}-dependent mitochondrial arrest still occurs.

PEX3Miro1 provides a novel way to ask whether SNPH is necessary for Ca^{2+}-dependent regulation of mitochondrial trafficking. Figure 3.1 shows a cartoon for how the PEX3Miro1 system can be utilized to look for Ca^{2+}-dependent disruption of mitochondrial trafficking, isolated from the presence of endogenous, mitochondrial SNPH. To study Miro1 Ca^{2+}-dependent regulation of mitochondrial trafficking and determine whether SNPH is necessary for the reported decrease in mitochondrial motility, we first set out to establish that the PEX3Miro1

system increased peroxisome trafficking. We tested this by overexpressing low levels of PEX3 control or PEX3Miro1 in COS-7 cells and treating them with the calcium ionophore calcimycin using the same protocol previously described in Wang et al., 2009 to increase intracellular Ca^{2+} concentration.

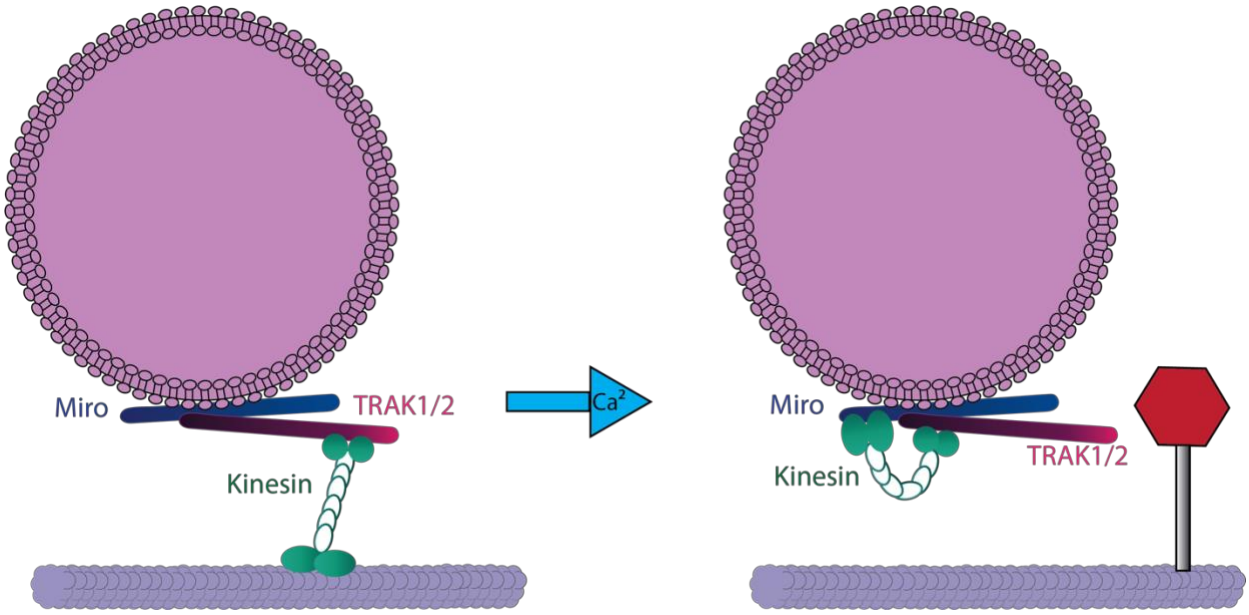


Figure 3.1 A model for studying Ca^{2+}-dependent disruption of motor-adaptor dependent trafficking using the PEX3Miro1 system.

Model showing how the PEX3Miro1 dependent recruitment of the motor-adaptor complex can be utilized to verify results from the Schwarz lab showing that increased intracellular calcium leads to decreased organelle trafficking. In this model, kinesin motor-domains are inactivated and directly interact with Miro1 following an increase in intracellular calcium.

We took movies of COS-7 cells which had been transfected with an mTurquoise-SRL peroxisomes marker, as well as the PEX3 control or PEX3Miro1 to capture peroxisome trafficking before and after treatment with the calcium ionophore, calcimycin (10uM in a saline solution containing 1.8mM Ca^{2+}). Peroxisome motility was scored by analyzing the mTurquoise-SRL peroxisome marker using a FIJI macro, described in Basu and Schwarz, 2020 that measures

the variance of pixels over time to give a readout of a total temporal variance for a given image. Although we saw an increase in peroxisome trafficking when low levels of PEX3Miro1 were overexpressed with the mTurquoise-SRL peroxisome marker, we only calculated a small difference between PEX3 control peroxisome motility and PEX3Miro1 peroxisome motility, making it complicated to interpret any Ca^{2+}-dependent motility defects (figure 3.2). We did however observe that treatment with calcimycin led to a small decrease in PEX3Miro1 peroxisome motility compared to untreated control. The lack of difference between PEX3 and PEX3Miro1 peroxisome motility is likely an effect of low expression of the PEX3Miro1 construct. Basu et al., *in preparation,* has demonstrated that the PEX3Miro1 construct increases peroxisome motility with higher expression of PEX3Miro1. Future studies with increased expression of PEX3 and PEX3Miro1 will be needed to reach any further conclusions on whether Ca^{2+}- dependent disruption of mitochondrial trafficking requires the SNPH anchor or if Ca^{2+} binding to Miro1 EF-hands is sufficient for this trafficking regulation to occur.

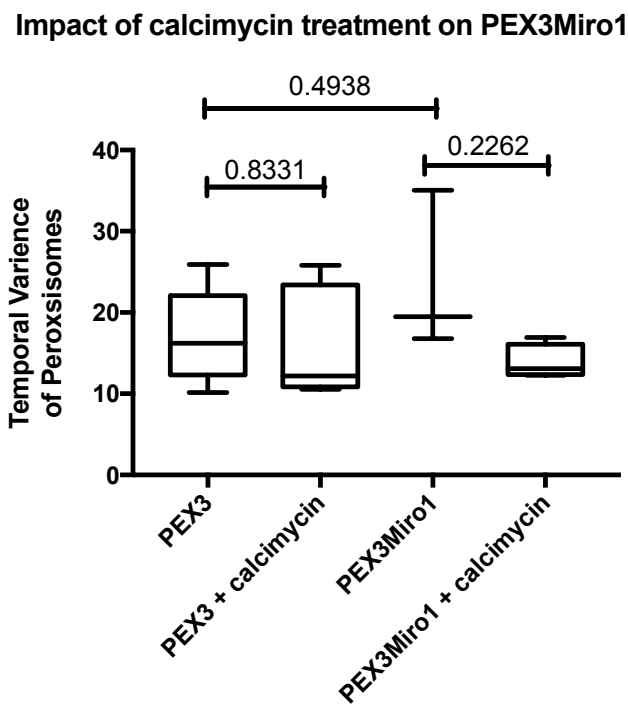


Figure 3.2 Low expression of PEX3Miro confers a small increase in peroxisome motility that is decreased with calcimycin treatment

Quantification of peroxisome temporal variance using the mTurquoise-SRL peroxisome marker channel with overexpression of PEX3 control or PEX3Miro1 in COS-7 cells. Treatment of COS-7 cells expressing PEX3 or PEX3Miro1 with calcium ionophore calcimycin

(10uM in a saline solution containing 1.8mM Ca^{2+}) did not show a significant difference in motility following calcimycin treatment as expected but also did not show a significant increase in motility from control PEX3 peroxisomes. The quantification is represented as 'Box and Whiskers' plots. The line indicates the median. For demonstrating statistical significance, two-tailed unpaired t-test with Welch correction was used. N=5 cells except for PEX3Miro where N=3. Images were collected in one experiment with no replicates.

PEX3Miro1 and TRAK1 are not sufficient for recruitment of SNPH to PEX3Miro1 peroxisomes

Although we did not see a significant PEX3Miro1-dependent increase in peroxisome motility or a strong effect of calcimycin treatment on peroxisome motility, we further investigated whether the PEX3Miro1 system is sufficient for co-localization of SNPH with PEX3Miro1 peroxisomes. SNPH has been described to directly interact with the kinesin motor, KIF5C, leading to the inhibition of the KIF5C motor domains (Chen and Sheng, 2009). It is unknown whether SNPH interacts with other proteins in the mitochondrial motor-adaptor complex. Figure 3.3 shows a cartoon for how the PEX3Miro1 system can be utilized to study whether the motor-adaptor complex is sufficient for co-localization of overexpressed SNPH with PEX3Miro1 peroxisomes in order to further investigate the mechanism by which SNPH regulates mitochondrial trafficking.

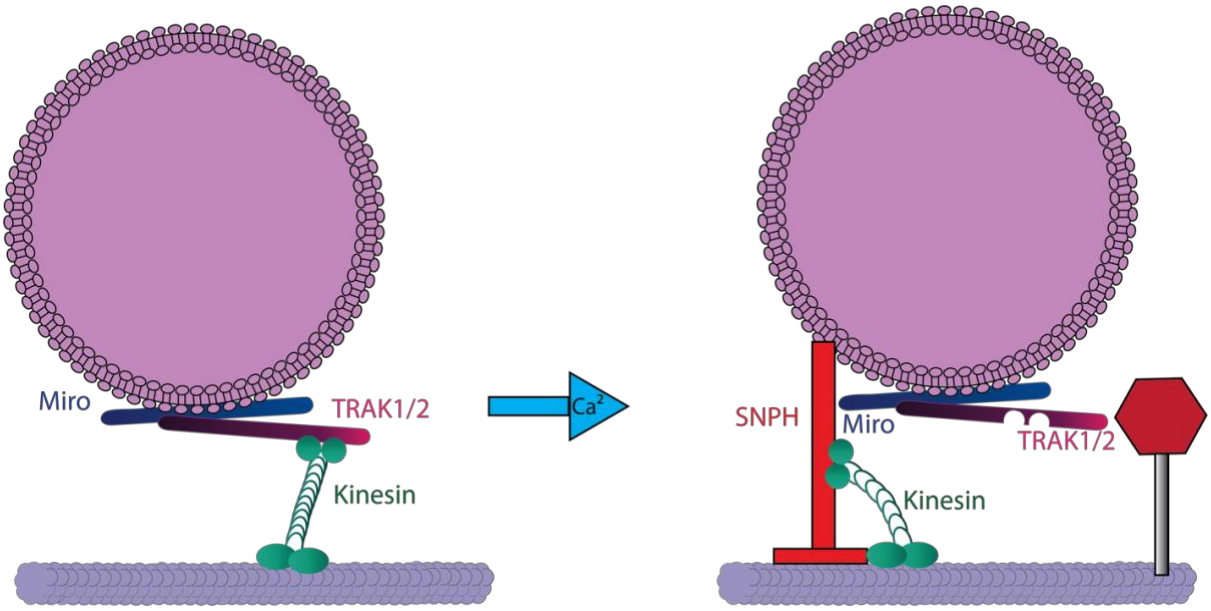


Figure 3.3 A model for studying SNPH-dependent anchoring of mitochondria
Model showing how PEX3Miro1 could be utilized to study how SNPH has been proposed to regulate mitochondrial motility by directly interacting with the kinesin motor. It is not known whether SNPH interacts with components of the motor-adaptor complex other than kinesin.

When we overexpress the mTurquoise-SRL peroxisome marker and SNPH-GFP with PEX3 control or PEX3Miro1, we find that overexpression of PEX3Miro1 is not sufficient for recruitment of SNPH-GFP to peroxisomes. Additionally, we find that the overexpression of PEX3Miro1 with myc-TRAK1 does not elicit co-localization of SNPH-GFP with PEX3Miro1 peroxisomes (figure 3.4) Interestingly, we did observe a peroxisome trafficking decrease when SNPH-GFP was overexpressed. It's unclear whether this is because SNPH is somehow directly altering PEX3Miro1-dependent peroxisome trafficking or if peroxisome trafficking has decreased as an indirect consequence of decreased mitochondrial trafficking upon the overexpression of SNPH (data not shown). We also found that overexpression of myc-TRAK1 with GFP-SNPH causes a re-distribution of PEX3Miro1 to what appears to be mitochondria or microtubules (figure 3.4) Further studies will be needed to be certain that syntaphilin does not interact with components of the motor-adaptor complex. In particular the recruitment of the kinesin motor to PEX3Miro1 peroxisomes will be essential for asking whether SNPH interacts with the kinesin motor to inhibit mitochondrial trafficking as proposed.

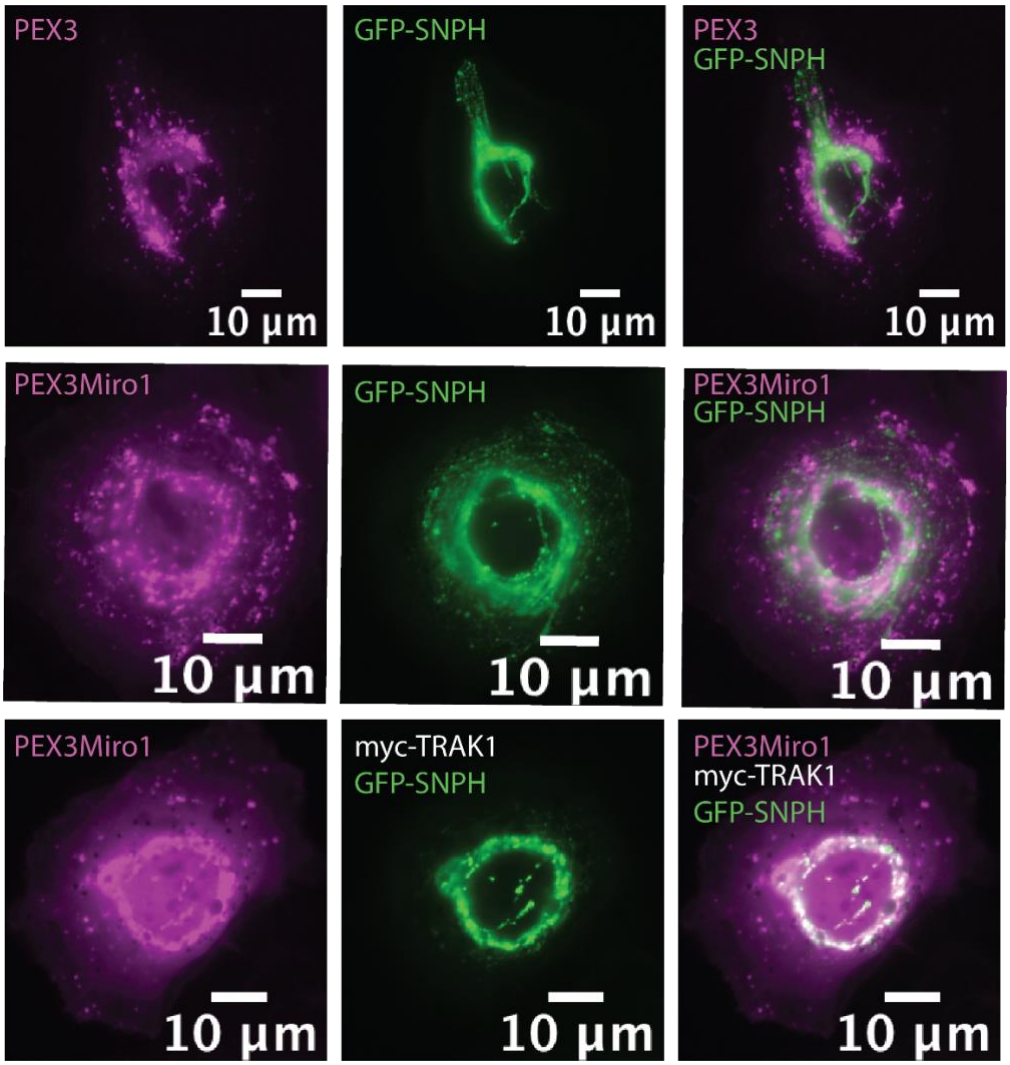


Figure 3.4 Syntaphilin does not directly interact with PEX3Miro1 or TRAK1 on PEX3Miro1 peroxisomes.

Overexpression of a GFP-tagged SNPH construct in COS-7 cells does not co-localize with overexpressed PEX3Miro1 on peroxisomes marked with an mTurquoise-SRL marker (signal not shown) with or without overexpression of myc-TRAK1 (signal not shown). Overexpression of myc-TRAK1 with GFP-SNPH causes a rearrangement of PEX3Miro1 localization.

Mfn2 localizes to PEX3 control peroxisomes and interacts with PEX3Miro1 in a TRAK1 dependent manner

Mitochondrial fusion is an essential process for regulating mitochondrial health and cellular homeostasis. Mitochondrial outer membrane fusion requires both mitofusin1 and mitofusin2 (Mfn1 and Mfn2) whereas inner membrane fusion is facilitated by Opa1. Both mitofusin proteins have previously been shown to interact with Miro1 and TRAK1 (Misko et al., 2019; Lee CA et al., 2018). Knockout of Mfn2 leads to decreased mitochondrial velocity and longer mitochondrial pausing events during trafficking (Misko et al., 2019). Recent work from Gerald Dorn's lab has also proposed that Mfn2 regulates the interaction between Miro1 and TRAK1 (unpublished).

Because the Mfn2 interaction with Miro1 is reported to regulate Miro1 interaction with TRAK1, we used the PEX3Miro1 system to ask whether Mfn2 co-localizes with PEX3Miro1 peroxisomes with and without the overexpression of myc-TRAK1. When we overexpressed the mTurquoise-SRL peroxisome marker and a YFP-MFN2 construct with either PEX3Miro1 or PEX3 control, we found that Mfn2 co-localizes with PEX3 control peroxisomes (figure 3.5A). Mfn2 has previously been reported to localize to ER membranes but has not been reported to localize to peroxisomes (Ngoh et al., 2012; Muñoz et al., 2013). In yeast, the mitofusin protein Fzo1 has been found to localize to peroxisomes and promote the formation of peroxisome-mitochondria contact sites (Shai et al., 2018). It is possible that this localization of Mfn2 on peroxisomes is just a result of overexpression of the Mfn2 construct. This is an interesting direction to further explore and it will be important to determine whether endogenous Mfn2 ever localizes to peroxisomes.

Although Mfn2 was shown to localize to peroxisomes with the PEX3 control, we compared the co-localization of Mfn2 to PEX3Miro1 peroxisomes with and without the

overexpression of myc-TRAK1 and used the co-localization macro described in chapter 2 to quantity the mean intensity of Mfn2 on peroxisomes. We found that when myc-TRAK1 is co-overexpressed with the SRL peroxisomes marker, PEX3Miro1, and Mfn2, there is a significant increase in the amount of Mfn2 co-localized to PEX3Miro1 peroxisomes (figure 3.5B). Importantly when we overexpressed PEX3 control with myc-TRAK1 and Mfn2, we did not observe an increased co-localization of Mfn2 to PEX3 control peroxisomes (figure 3.5B). Interestingly, representative images also show that the overexpression of myc-TRAK1 with PEX3 or PEX3Miro1 seems to drastically change the localization of Mfn2 in the cell. Without myc-TRAK1 overexpression, there is a strong localization of Mfn2 to a perinuclear mitochondrial network. With the overexpression of myc-TRAK1, Mfn2 appears to be more disperse throughout the cell on what seem to be more distributed mitochondria (figure 3.5A).

Together these results show that TRAK1 and Mfn2 co-localize on PEX3Miro1 peroxisomes and suggests that an interaction between TRAK1 and Mfn2 plays a role in the localization of Mfn2.

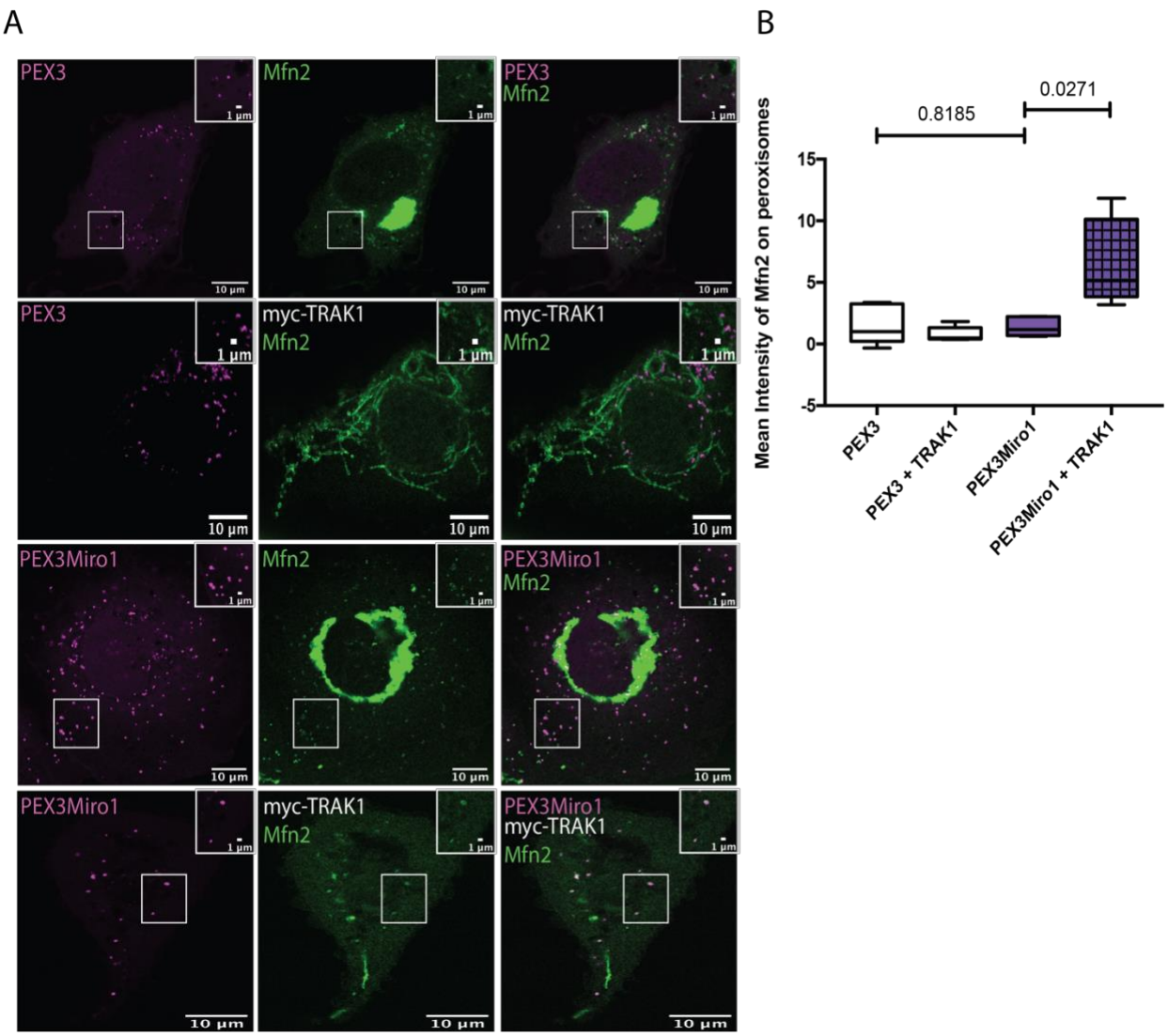


Figure 3.5 TRAK1 increases Mfn2 co-localization to PEX3Miro1 peroxisomes.

Overexpression of myc-TRAK1 increases co-localization of Mfn2 with PEX3Miro1 peroxisomes

- *A) Overexpression of mturquoise-SRL peroxisome marker (signal not shown) 6xhis-mRFP-PEX3 control or 6xhis-mRFP-PEX3Miro1 (signal shown in magenta) with YFP-Mfn2 shown in green, with or without the overexpression of myc-TRAK1(signal not shown) in COS-7 cells. Cells were fixed 48 hours after transfection.*
- *B) Quantification of YFP- Mfn2 with PEX3 control or PEX3Miro1 peroxisomes in the presence or absence of overexpressed myc-TRAK1. Co-localization is shown as the mean intensity of Mfn2 signal on PEX3 or PEX3Miro1 peroxisomes. The quantification is represented as 'Box and Whiskers' plots. The line indicates the median. For demonstrating statistical significance, two-tailed unpaired t-test with Welch correction was used. Outliers are represented as single plot points and were included in all statistical calculations. N=5 cells from 1 experiment.*

Overview of findings

In this chapter, I describe two ways that the PEX3Miro1 system has been used to test models for regulation of the mitochondrial motor-adaptor complex. There are complications with using the PEX3Miro1 system, including figuring out the appropriate PEX3Miro1 expression levels to elicit a PEX3Miro1-dependent increase in peroxisomes motility and ensuring that localization of proteins are not overexpression artifacts. However, the PEX3Miro1 system has proven to be a useful tool for studying Miro1 protein interactions. Using PEX3Miro1, we collected preliminary data that suggests that PEX3Miro1 can be utilized for studying the Ca^{2+}-dependent regulation of mitochondrial trafficking. Since this work, Basu et al., *in preparation*, has established a protocol for initiating increased motor-adaptor dependent motility on peroxisomes using the PEX3Miro1 system, which can be utilized to continue this work. We found that PEX3Miro1 and TRAK1 are not sufficient for co-localization of SNPH to peroxisomes. It is still not fully understood how SNPH interacts with the motor-adaptor complex and further work will need to be done to elucidate the mechanism of SNPH-dependent mitochondrial anchoring.

While using the PEX3Miro1 system to study a proposed model for Mfn2 regulation of the interaction between Miro1 and TRAK1, we found that overexpressed YFP-Mfn2 localized to peroxisomes with the overexpressed PEX3 control. To determine whether this is an artifact of overexpression or a real localization of Mfn2 on peroxisomes, further work will need to be done to look for the co-localization of endogenous Mfn2 with a peroxisomal marker. We also found that Mfn2 localizes to PEX3Miro1 peroxisomes at a similar level as the PEX3 control and that this co-localization of Mfn2 to peroxisomes drastically increases upon overexpression of myc-TRAK1. This is preliminary data and will need to be repeated. Overall, we show that the PEX3Miro system is a useful tool to orthogonally study protein interactions with components of

the motor-adaptor complex and regulation of motor-adaptor complex dependent organelle trafficking.

Chapter 4.
Extended discussion and future experiments

4.1 Introduction

For my thesis work in the Schwarz lab, we set out to understand the function of the Miro1 N-terminal and C-terminal GTPase domains. To do this, we used a method in which the Miro1 protein is directed to peroxisomes by being linked to the transmembrane domain of the PEX3 peroxisomal membrane protein. This method allowed us to study Miro1 function in a way that's isolated from endogenous, mitochondrial Miro. The PEX3Miro system opened up the possibility of asking a number of different questions about how the mitochondrial motor-adaptor complex is regulated and how it interacts with mitochondria-associated proteins. As a result, we have uncovered a mechanism for how Miro1's N-terminal GTPase domain regulates the assembly of the mitochondrial motor-adaptor complex. We have also used the PEX3Miro1 system to ask questions about how the motor-adaptor complex assembles. Using PEX3Miro1, we have been able to test hypotheses about the requirement of a TRAK protein for the recruitment of kinesin to the motor-adaptor complex, as well as hypotheses about the functional differences between TRAK1 and TRAK2 in their ability to interact with kinesin. In addition, the PEX3Miro system has been a useful tool to explore the functional differences between Miro1 and Miro2. We have found that Miro2 does not interact with components of the motor-adaptor complex in either it's wild-type state or with its nGTPase or cGTPase locked in constitutively active or inactive states. Overall, this work has contributed insights into the regulation of mitochondrial motility.

PEX3Miro1 as a tool for studying the components of the motor-adaptor complex

The PEX3Miro1 system serves as a useful tool for recruitment of the motor-adaptor complex. In this thesis, I was able to recruit the adaptor proteins TRAK1 and TRAK2, and the plus-end directed motor protein, kinesin, to peroxisomes. One important future direction of study is to ask the how the minus-end directed motor, dynein, interacts with the motor-adaptor complex. We

have attempted to use the overexpression of the dynein-dynactin complex protein, P150-glued, to study dynein co-localization with PEX3Miro1. Although we were able to see some co-localization of PEX3Miro1 peroxisomes with P150-glued, the majority of the P150-glued localization was on microtubules. It will be interesting to continue to study how components of the dynein-dynactin complex interact with PEX3Miro1 peroxisomes and whether the adaptor proteins TRAK1/2 are necessary for dynein's interaction with Miro1. It will also be interesting to compare how the TRAK1/2 adaptors might differentially interact with kinesin or dynein to regulate plus-end and minus-end directed trafficking in both COS-7 cells and hippocampal neurons.

One issue with the PEX3Miro1 system is the mitochondrial localization of PEX3Miro1 with high levels of overexpression. Although we also find that the PEX3 control localizes to mitochondria with high expression levels, we cannot rule out that PEX3Miro1 might dimerize with endogenous Miro1 on mitochondria. It will be important to further control for this and measure the intensity of PEX3Miro1 expression that co-localizes with a mitochondrial marker compared to PEX3 control to fully understand how this system works so that we can interpret our results with all potential pitfalls in mind.

We were surprised to find that the transmembrane domain of PEX3 was sufficient for mitochondrial localization of the overexpressed control construct. Throughout this thesis we found that a number of peroxisome membrane associated proteins localized to mitochondria upon overexpression. While testing peroxisome marker antibodies we also found the localization of some endogenous peroxisome membrane associated proteins localized to mitochondria. The study of membrane dynamics between peroxisomes and mitochondria will be an interesting field

to follow and developments in this area of research will be important to consider for the continued use of the PEX3Miro1 system.

Further complicating the PEX3Miro1 system is the finding that some isoforms of the Miro1 protein are found to localize to peroxisomes. The function of Miro1 on peroxisomes is not known although it seems to play a role in peroxisomal trafficking. One important factor to consider in studying proteins that seem to interact with both peroxisomes and mitochondria is that mitochondrial and peroxisomal trafficking dynamics might be dependent on one another, meaning that altered peroxisome trafficking as an effect of Miro1 knockdown or Miro1 overexpression could be a reflection of an increase or decrease in mitochondrial trafficking and this should be controlled for by measuring the trafficking dynamics of both organelles side by side. There are large discrepancies in the reports of endogenous Miro1 on peroxisomes and more work will be needed to understand which variants of Miro1 are endogenously localized to peroxisomes and what role Miro1 plays on peroxisomes.

Overall, we have found a way to study the PEX3Miro1 system in isolation of these pitfalls and have been able to utilize the PEX3Miro1 system to reveal a mechanism for Miro1 GTPase domain regulation of the motor-adaptor complex, as well as identify functional differences between TRAK1 and TRAK2 and Miro1 and Miro2.

Miro1 GTPase domains regulate the motor-adaptor complex

In this thesis, we have utilized the PEX3Miro1 system to study the functional regulation of the Miro1 GTPase domains. We found that the Miro1 nGTPase regulates the interaction of PEX3Miro1 with TRAK1 and TRAK2, which impacts the recruitment of the kinesin motor to the motor-adaptor complex. In addition, we discovered that the Miro1 cGTPase plays a role in

the regulation of the motor-adaptor complex. The cGTPase constitutively active mutation is most similar to wild-type PEX3Miro1 in its ability to interact with TRAK and kinesin. Although the nGTPase constitutively active mutation is sufficient for the assembly of the motor-adaptor complex, this interaction is greatly enhanced when the Miro1 cGTPase is also in the constitutively active mutation. This result suggests that there's some coordination between the Miro1 nGTPase and cGTPase in the regulation of the motor-adaptor complex that should further be studied. Because no one has been able to crystalize the structure of the full Miro1 protein, it is currently unknown how the two Miro1 GTPase domains might interact with each other and this will be an interesting area of future study. It will also be interesting to continue to utilize the recently published full length purified Miro1 protein to study how the GTPase domains are regulated (Heinrichs et al., 2020).

Co-Immunoprecipitation experiments with PEX3Miro1 for studying the function of the PEX3Miro1 nGTPase

In an attempt to orthogonally show that the PEX3Miro1 N-terminal GTPase regulates the assembly of the mitochondrial motor-adaptor complex, we have tried to demonstrate the differences between constitutively active and constitutively inactive nGTPase mutants of PEX3Miro1 by co-immunoprecipitation of the PEX3Miro1 construct. This experiment has proven to be exceptionally difficult for several reasons. First and foremost, we have attempted to co-immunoprecipitate a protein complex which has been artificially re-directed to peroxisomes. Secondly, we hypothesized that this co-immunoprecipitation would be able to retain GTPase specific interactions and be reflective of our co-localization results in COS-7 cells. Third, we hypothesized that PEX3Miro1 would not dimerize with endogenous Miro1 in a way that would

cloud our results. Throughout the many experiments done, we ran into all three of the aforementioned issues.

One issue that we consistently ran into, was that the PEX3Miro wild-type construct seemed to be less stable than the PEX3Miro GTPase mutants. We were never able to enrich for the same quantity of PEX3Miro1 wild-type in co-immunoprecipitation experiments as we were the GTPase mutants, despite similar amounts of overexpressed PEX3Miro1 constructs in our starting lysates. To get around this, we spent a lot of time optimizing our co-immunoprecipitation protocol to lead to improved enrichment of PEX3Miro constructs. This optimization included altering the lysis buffer, capture beads, antibodies, and incubation times. Overall, we found that a combination of using dynabeads and a monoclonal RFP antibody along with an increased concentration of $MgCl_2$ in our lysis buffer led to the best co-immunoprecipitation outcomes for the wild-type PEX3Miro1 construct and its association with TRAK1 and kinesin. Even though the enrichment of PEX3Miro1 improved with these conditions, we struggled with seeing the GTPase specific differences that were so obvious and repeatable by co-localization studies.

Throughout the many iterations of the PEX3Miro1 co-immunoprecipitation experiment, we ran into a consistent issue of our co-immunoprecipitation results not being reflective of the results we saw by co-localization. By co-localization, we were able to consistently see a difference in our PEX3Miro1-nGDP and PEX3Miro1-nGTP mutants' ability to facilitate the co-localization of PEX3Miro1 peroxisomes with components of the motor-adaptor complex. In our co-immunoprecipitation experiment, we saw by Western blot that the wild-type PEX3Miro1 consistently interacted with the lowest quantity of TRAK and kinesin. We also found that the PEX3Miro1 nGTPase GDP mutant, which does not co-localize with any TRAK1 or kinesin,

could interact with both proteins by co-immunoprecipitation. When the amount of TRAK1 and kinesin that co-immunoprecipitated with the PEX3Miro1 nGTPase GDP mutant was quantified, we found that the nGTPase GDP mutant interacted with more TRAK1 and kinesin than the PEX3Miro1 nGTPase constitutively active mutant (GTP mutant) or wild-type PEX3Miro1 (figure 4.1) We formed many hypotheses to explain this difference. These hypotheses included that 1) Our lysis buffer did not contain the correct ion concentration for a GTPase specific interaction to occur 2) That the interaction of PEX3Miro1 with the motor-adaptor complex was reliant on a bound GTP or GDP or that a GTPase specific interactor such as a GEF or a GAP was required to regulate the Miro1 nGTPase and that the integrity of this factor was lost in our co-immunoprecipitation experiments. To further investigate this, future experiments will be done in the presence of GDP or GMPPNP to look for GDP and GTP specific differences in the ability of wild-type PEX3Miro1 to interact with the motor-adaptor complex. It will also be interesting to put this hypothesis to test by overexpressing the GAPs and GEFs that are reported to regulate Miro1 and test whether they affect the ability of PEX3Miro1 to interact with the motor-adaptor complex.

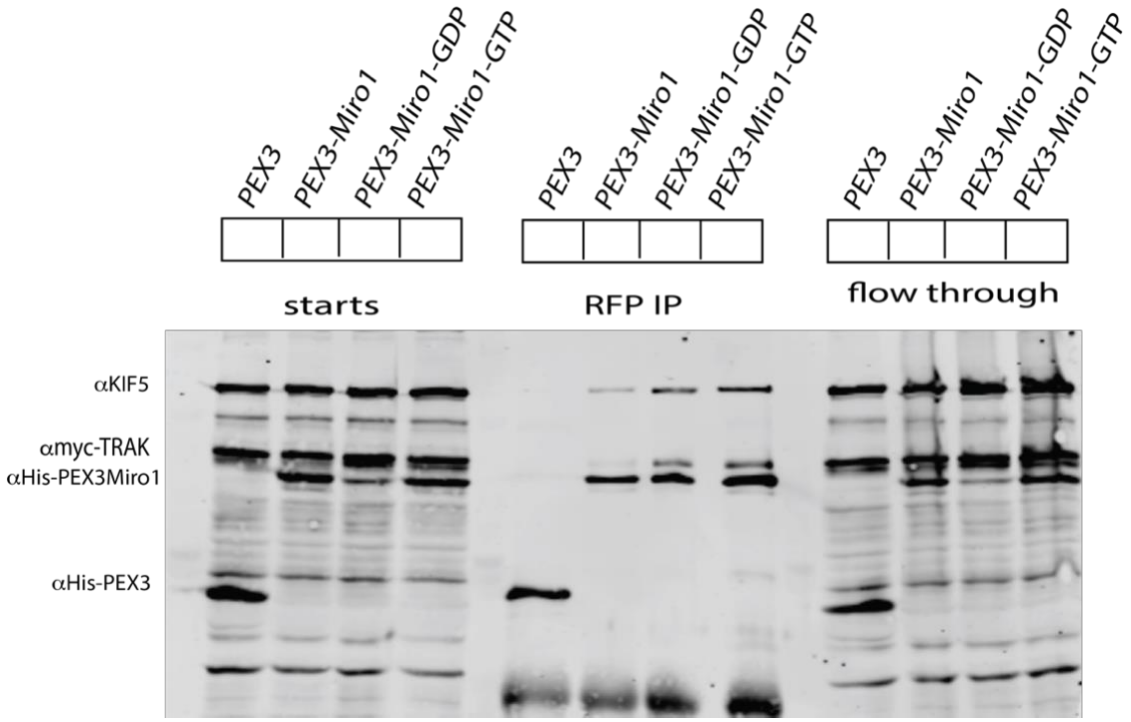


Figure 4.1 PEX3Miro1 nGTPase mutant co-immunoprecipitation of TRAK1 and KIF5C
Overexpressed myc-TRAK1 and KIF5C co-immunoprecipitate with overexpressed 6xhis-mRFP-PEX3Miro1 compared to PEX3Miro1 nGTPase mutants and PEX3 control in HEK293T cells.

A) Western blot showing co-immunoprecipitation of overexpressed myc-TRAK1 and mCitrine-KIF5C with overexpressed 6xhis-mRFP-PEX3Miro1. HEK293T cells were transfected with 6xhis-mRFP-PEX3Miro1 or 6xhis-mRFP-PEX3 control plasmid and lysates were collected 48 hour later using Pierce IP lysis buffer. Western blot was blocked with TBST (.1% triton x-100) and stained with 6xHIS antibody from Thermo Fisher Scientific (catalogue number: MA1-21315), Myc antibody from EMD Milipore (catalogue 05-724), and KIF5 antibody from EMD Milipore (catalogue number MAB1614)

Finally, for our PEX3Miro1 system to work in immunoprecipitation experiments would require that all the PEXMiro1 in a cell is in a complex like that on peroxisomes. We knew, however, that some PEX3Miro1 was also present on mitochondria where it could dimerize with a significant amount of endogenous, mitochondrial Miro1. Because the volume of peroxisomes in these cells is far less than that of mitochondria, even if the density of the PEX3Miro1 is lower on mitochondrial membrane, that mitochondrial pool might still greatly exceed the pool on peroxisomes. If PEX3Miro1 on mitochondria forms complexes with endogenous wildtype Miro,

it may interact with TRAK1 and KIF5C, independent of the nGTPase state of the PEX3Miro1component. This is the most likely explanation of the disconnect between the localization studies and the immunoprecipitation. Indeed, we found significant levels of co-immunoprecipitation of endogenous Miro1 with PEX3Miro1, but not with the PEX3 control (figure 4.2). When we looked for the interaction of endogenous Miro1 with PEX3Miro1 and the PEX3Miro1 nGTPase mutants, we found that the PEX3Miro1 nGTPase mutants also interacted with a significant amount of endogenous Miro1 and that the amount of endogenous Miro1 that co-immunoprecipitated with PEX3Miro1 seemed to impact the amount of TRAK1 and KIF5C that were co-immunoprecipitated (figure 4.3). Quantification of the amount of endogenous Miro1, TRAK1, and KIF5C co-immunoprecipitated with PEX3Miro1, showed a strong correlation between the amount of endogenous Miro1 co-immunoprecipitated with the amount of TRAK1 and KIF5C co-immunoprecipitated, which can also be observed by eye (Quantification data not shown). This result significantly clouds our ability to interpret PEX3Miro1 co-immunoprecipitation experiments with the PEX3Miro1 nGTPase mutants, and we will need to take the alternative approach of studying PEX3Miro1 in a Miro1 null background or using in vitro binding studies.

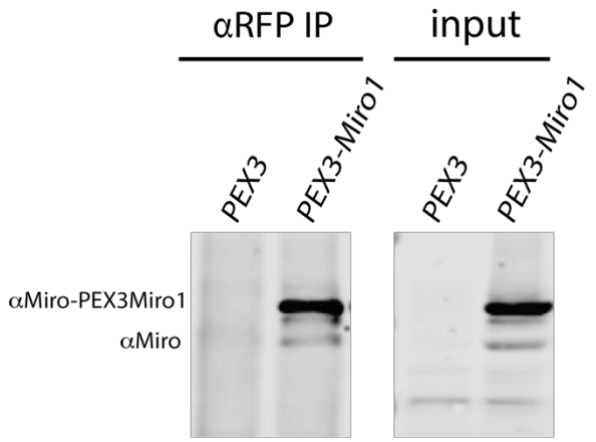


Figure 4.2 Co-immunoprecipitation of endogenous Miro1 with PEX3Miro1.
Endogenous Miro1 co-immunoprecipitated with overexpressed 6xhis-mRFP-PEX3Miro1 in HEK293T cells.

A) Western blot showing co-immunoprecipitation of endogenous Miro1 with overexpressed PEX3Miro1 or PEX3 control. HEK293T cells were transfected with 6xhis-mRFP-PEX3Miro1 or 6xhis-mRFP-PEX3 control plasmid and lysates were collected 48 hour later using Pierce IP lysis buffer. The Western blot was blocked in TBST (.1% triton-100) and stained with RHOT1 (Miro1) antibody from Aviva Biosystems (catalogue number: ARP44817_P050).

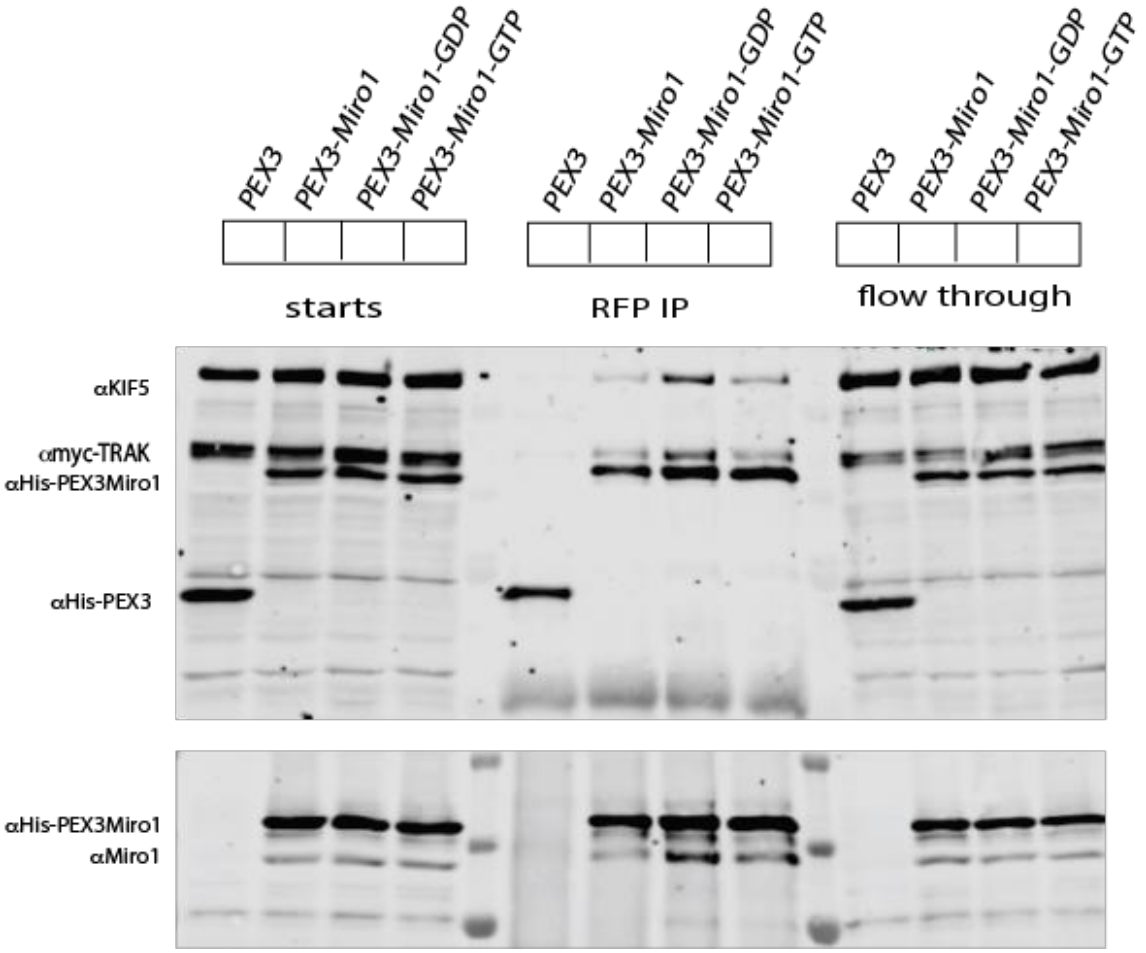


Figure 4.3 PEX3Miro1 nGTPase mutant co-immunoprecipitation of endogenous Miro1.
Overexpressed myc-TRAK1 and KIF5C co-immunoprecipitate with overexpressed 6xhis-mRFP-PEX3Miro1 compared to PEX3Miro1 nGTPase mutants and PEX3 control in HEK293T cells. Endogenous Miro1 is found to co-immunoprecipitate with PEX3Miro1 and PEX3Miro1 nGTPase mutant constructs.

A) Western blot showing co-immunoprecipitation of overexpressed myc-TRAK1, mCitrine-KIF5C, and endogenous Miro1 with overexpressed 6xhismRFP control, 6xhis-mRFP-PEX3Miro1, and 6xhis-mRFP-PEX3Miro1 mutants. HEK293T cells were transfected with 6xhis-mRFP-PEX3Miro1 constructs or 6xhis-mRFP-PEX3 control plasmid and lysates were collected 48 hour later using Pierce IP lysis buffer. Western blot stained with 6xhisHIS antibody from Thermo Fisher Scientific (catalogue number: MA1-21315), Myc antibody from EMD Milipore (catalogue 05-724), KIF5 antibody from EMD Milipore (catalogue number MAB1614), and RHOT1 (Miro1) antibody from Aviva Biosystems (catalogue number: ARP44817_P050)

A proposed mechanism for the Miro1 N-terminal GTPase regulation of motor-adaptor complex assembly

In this thesis we have found that the Miro1 N-terminal GTPase can regulate the assembly of the mitochondria motor-adaptor complex. In contrast to previously proposed mechanisms for the regulation of the mitochondrial motor-adaptor complex, we find that Miro1 nGTPase regulates the interaction between Miro1 and TRAK. We know of no other example of the complete disassembly of the motor-adaptor complex. In figure 4.4, we propose a model for how the Miro1 N-terminal GTPase domain regulates the assembly of the mitochondrial motor-adaptor complex. Although this GTPase specific mechanism provides a model which fits in with the previous reports of Miro1 nGTPase regulation of mitochondrial trafficking and localization, we still do not understand when the Miro1 nGTPase is used to regulate the assembly of the motor-adaptor complex.

One attractive model for how the Miro1 nGTPase is regulated is proposed in Suzuki et al., 2014 where the VopE *Vibrio Cholera* Type III secretion system effector has been described as a GAP that regulates the Miro1 nGTPase domain. In this model, the authors propose that VopE acts as a GAP to regulate Miro1 mitochondrial localization. They find that interaction with VopE leads to a rearrangement of the mitochondrial network. When they infect cells with *Vibrio Cholera,* the mitochondrial network appears to collapse, leading to a perinuclear localization of mitochondria. When VopE is expressed in these cells, the mitochondrial network leaves this perinuclear localization and mitochondria become more dispersed throughout the cell. This model fits nicely with our finding that the Miro1 nGTPase regulates the assembly of the motor-adaptor complex, attaching mitochondria to microtubules through the kinesin motor. With this result in mind, a model where the GTP bound status of the Miro1 nGTPase is changed by interaction with VopE

and the subsequent hydrolysis of the bound Miro1 nGTP, could lead to the disassembly of the motor-adaptor complex, leading to a relocalization of mitochondria away from microtubules. Suzuki et al., propose that VopE takes advantage of the Miro1 nGTPase to inhibit the cellular stress signaling that could activate the innate immune system upon infection with *Vibrio Cholera*. While this is a very interesting mechanism for *Vibrio Cholera* infection, we are curious how the Miro1 nGTPase might be regulated by GEFs and GAPs in more typical cellular scenarios and will continue to look for GEFs and GAPs that regulate this interaction in an attempt to better understand when the Miro1 GTPase is used to regulate motor-adaptor complex assembly.

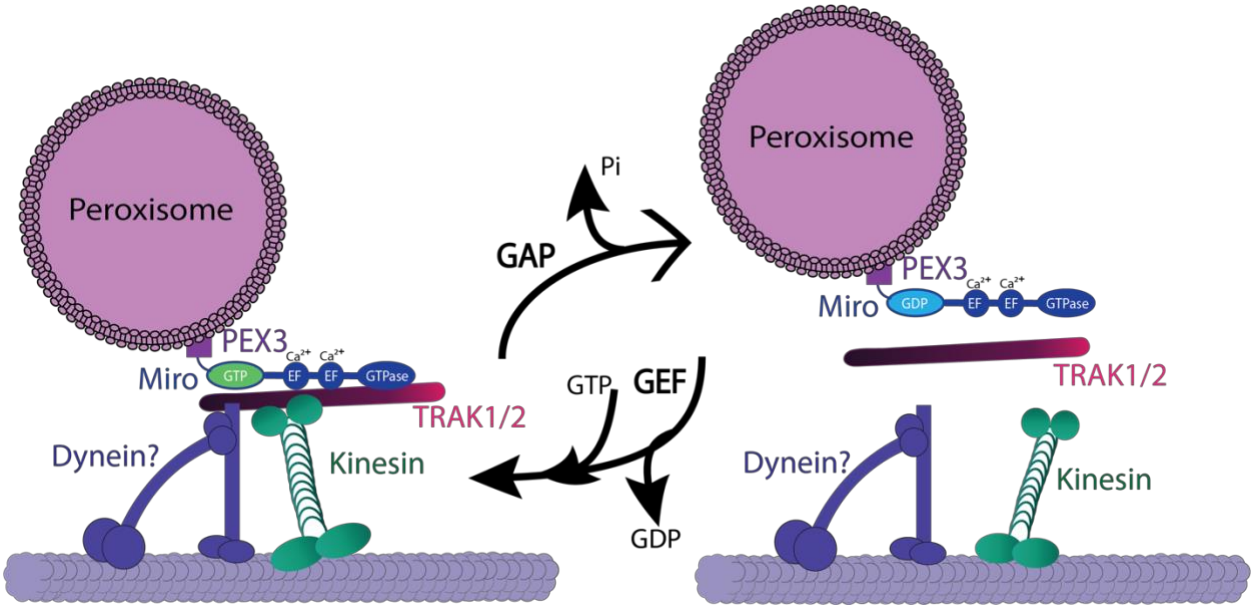


Figure 4.4 Model for motor-adaptor complex assembly regulation by PEX3Miro1 nGTPase domain

When the PEX3Miro1 nGTPase is bound to GTP, the motor-adaptor complex is assembled with TRAK1/2 and kinesin-1 associating with PEX3Miro1. We do not yet know whether the nGTPase of Miro1 regulates the interaction of the dynein motor with PEX3Miro1. When the GTP bound to PEX3Miro1 nGTPase is hydrolyzed and the nGTPase is bound to GDP, the motor-adaptor complex disassembles with TRAK1/2 and kinesin losing interaction with PEX3Miro1. We do not know whether this GTPase dependent regulation also effects the ability of dynein to interact with PEX3Miro1.

Miro1 and Miro2 differ

In this thesis we use the PEX3Miro system to compare Miro1 and Miro2. We were surprised to find that PEX3Miro2 was unable to recruit the TRAK adaptor or the kinesin motor to peroxisomes. Further experiments will be needed to elucidate this difference between Miro1 and Miro2 and to understand what the role of Miro2 is on mitochondria. It will be interesting to study the localization of the endogenous Miro1 and Miro2 to understand whether Miro1 and Miro2 are both localized to all mitochondria or if they localize to different populations of mitochondria. If they do localize to different populations of mitochondria, it will be interesting to determine whether Miro1 and Miro2 regulate mitochondrial trafficking differently or if Miro1 and Miro2 localization might regulate the interaction between mitochondria and different organelles, like the ER membrane and peroxisomes.

Conclusions

Mitochondrial trafficking is highly regulated with many different components involved in the regulation of mitochondrial localization, directionality, and velocity. In this thesis we describe a novel mechanism for the regulation of mitochondrial motility. The function of the Miro1 GTPase domains has long been known to be important for mitochondrial trafficking and cellular health. Our proposed model for the regulation of the Miro1 nGTPase domain fits nicely with previous studies and shows a functional mechanism for why mutations in the nGTPase domain locking Miro1 nGTPase in either a constitutively inactive GDP like state or constitutively active GTP like state leads to changes in mitochondrial motility and distribution. Although the described mechanism for regulation of the Miro1 GTPase domains offers an explanation for these mitochondrial motility and localization phenotypes, it will be important to continue to study

when the Miro1 GTPase domains regulate the assembly of the motor-adaptor complex. It will also be interesting to continue to study the extent of the Miro1 GTPase domains ability to regulate known Miro1 interactions with other proteins. In addition, it will be important to identify how the Miro1 GTPase domains are endogenously regulated. Understanding how the assembly of the mitochondrial motor-adaptor complex is regulated and how this relates to mitochondrial trafficking is essential for understanding how mitochondria are regulated in healthy cells and how the disruption of mitochondrial trafficking can relate to disease.

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