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Miro: A molecular switch at the center of mitochondrial regulation

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Abstract

The orchestration of mitochondria within the cell represents a critical aspect of cell biology. At the center of this process is the outer mitochondrial membrane protein, Miro. Miro coordinates diverse cellular processes by regulating connections between organelles and the cytoskeleton that range from mediating contacts between the endoplasmic reticulum and mitochondria to the regulation of both actin and microtubule motor proteins. Recently, a number of cell biological, biochemical, and protein structure studies have helped to characterize the myriad roles played by Miro. In addition to answering questions regarding Miro's function, these studies have opened the door to new avenues in the study of Miro in the cell. This review will focus on summarizing recent findings for Miro's structure, function, and activity while highlighting key questions that remain unanswered.

Keywords: Miro, mitochondria, organelle contact sites, microtubule transport, GTPase, neurodegeneration

Acronyms

- **cGTPase** - C-terminal GTPase domain of Miro
- **ER** - Endoplasmic Reticulum
- **ERMES** - ER-mitochondria encounter structure
- **ERMCS** - ER-mitochondria contact site
- **GAP** - GTPase activating protein

- **GEF** - GTPase exchange factor
- **HDAC6** - histone deacetylase 6
- **iPSCs** - induced pluripotent stem cells
- **LRRK2** - leucine-rich repeat kinase 2
- **MICOS** - Mitochondrial cristae organizing structure
- **MCU** - mitochondria calcium uniporter
- **Miro** - Mitochondrial Rho GTPases
- **nGTPase** - N-terminal GTPase domain of Miro
- **OMM** - Outer-mitochondrial membrane

Introduction

Mitochondria represent a key component of the cell, sitting at the interface of cellular metabolism, energy production, and cell death. These diverse functions of mitochondria require a host of proteins that function within mitochondria as well as on the outer mitochondrial membrane. Continued proteomic efforts have mapped 1,000+ components to the mitochondria,[1,2] despite the fact that the mitochondrial genome only encodes for 13 genes. This indicates that a significant percentage of cellular factors participate in mitochondrial biology.

Over the past twenty years, a growing body of evidence has implicated the *mi*tochondrial *R*ho GTPase (Miro) family of proteins as regulators of mitochondrial cellular function. Originally classified as a member of the Rho family, human Miro1 and Miro2[3] (RHOT1 and RHOT2, respectively) are now considered to be founding members of its own family. The Miro family are atypical GTPases within the Ras superfamily, composed of two GTPase domains flanking two EF-hand motifs [Fig. 1(A)]. These domains indicate that the Miro family likely exploits allostery in order to regulate cellular processes. Indeed, over

the course of twenty years, numerous studies have implicated Miro activity as playing important roles through mutagenesis of these domains.

In this review, we will highlight recent publications that connect the Miro family to diverse cellular processes. Considering that the majority of studies on the Miro family have focused on Miro1, the term “Miro” will refer to Miro1 unless otherwise noted. We will begin with a discussion about the role that Miro family members play with inter-organelle contacts as they stitch together a number of cell biology processes. Then, we will narrow our focus onto how Miro family members facilitate cytoskeletal trafficking with a particular emphasis on recent biochemical and molecular characterizations.

Miro: an atypical GTPase

Members of the Miro family are single-pass, type IV transmembrane proteins, consisting of two GTPase domains flanking two calcium-binding domains (EF-hands) with a C-terminal tail-anchored in the mitochondrial outer membrane[3–5] [Fig. 1(A)]. Even though the sequences of the GTPases and EF-hand motifs vary, the overall domain architecture of Miro is conserved throughout single-celled eukaryotes to plants and animals.

A hallmark feature of Miro is the presence of two GTPase domains [Fig. 1(A)]. Typically, small GTPases like Miro act as molecular switches and cycle between two states: an active, GTP-bound state and an inactive, GDP-bound state.[6] Like other Ras family G-proteins, G1, G4, and G5 loop sequence motifs are conserved in Miro.[7] However, Miro GTPases vary from other members of the Ras superfamily in the Switch I/II regions. In general, Switch I regions are involved in the stabilization of nucleotide, whereas Switch II is involved in the regulation of nucleotide hydrolysis. The Switch regions for both the C-terminal (cGTPase) and N-terminal (nGTPase) domains of Miro1 are shown in Figure 1(B). For Miro2, these regions vary slightly in the C-terminal GTPase domain, with Switch

I corresponding to residues 446-455 and Switch II comprising residues 472-489.[7] Typically, in the GTP-bound state, the surface loops are known as Switch I (G2) and Switch II (G3) and exist in close proximity to one another.[8,9] However, the Rho-conserved DxxG Switch II motif is absent from Miro1.[10] Other differences in these regions are variations in the key catalytic residues of Ras (Y32, T35, G60, and Q61).[7] Together, current research demonstrates that Miro is a distinct protein, with unique GTPase domains.

As a small G-protein with two GTPases, both GTPase domains may act as potential regulators of Miro activity. In order to dissect the role of the GTP state of Miro, characteristic mutations are typically introduced into Miro's nGTPase or cGTPase in order to stabilize Miro in the GTP or GDP form. For the nGTPase, GTP is stabilized via the mutation P13V and whereas the GDP form is promoted by T18N[3,11,12] [Fig. 1(B)]. For the cGTPase, the GTP and GDP-locked forms correspond to V425/V427 and N430/N432 for Miro1/2, respectively[3] [Fig. 1(B)]. While some authors[3] have described the N432 mutant in Miro2 to be GDP-locked, others have described this mutant to behave similarly to wild-type Miro.[13]

While a full-length structure of Miro has yet to be determined, there are two separate structural studies on Miro comprising a C-terminal fragment (MiroS: EF1-EF2-cGTPase)[5,8] and a recent structure of the nGTPase[9] [Fig. 1(C)]. Previous work on MiroS indicated 1) the EF-hands form a tightly packed interaction with the cGTPase and 2) Ca^{2+} binding to the EF-hands did not cause dramatic structural changes. Moreover, in the structure of MiroS, Miro's cGTPase was found to be bound to GDP, GDP-Pi, or GMPPCP in conjunction with either Ca^{2+} or Mg^{2+},[5,8] indicating that both EF-hands and the cGTPase can engage ligands simultaneously.

Recently, the structure of the isolated nGTPase was determined using X-ray crystallography.[9] Surprisingly, despite the absence of nucleotide during protein purification and crystallization, the nGTPase crystallized with GTP bound to the active site.[9] This suggests that the nGTPase alone may not be sufficient for GTP hydrolysis. Interestingly, both nGTPase and cGTPase structures both showed the presence of dimers within the asymmetric unit of the structure, which suggests that Miro may be able to exist in higher-order structures. However, size-exclusion chromatography and small-angle X-ray scattering indicated that these constructs were monomers in solution, allowing the authors to conclude that Miro does not dimerize in solution. Finally, recent small-angle X-ray scattering studies suggest that the nGTPase may be loosely tethered to the MiroS.[9] This suggests that the nGTPase may exist in multiple conformations or that the nGTPase is capable of interacting with MiroS.

Throughout the review, the structure of Miro will serve as a framework with which to understand the diverse cellular activities. This will involve mutations that alter the GTP state, phosphorylation sites, and calcium-binding. To help the reader, we have constructed an *in silico* model of Miro based on superimposing Miro's nGTPase[9] with the EF1-EF2-cGTPase structure[5] [Fig. 1(C)].

Miro facilitates inter-organelle interactions with cytoplasmic machinery

Before discussing Miro's well-known role in cytoskeletal transport of mitochondria, we will first discuss the role that Miro plays in facilitating mitochondrial interactions with other cellular compartments. To date, Miro has been implicated in mediating contact and interaction with the endoplasmic reticulum (ER), peroxisomal movement, and mitochondrial shape transition necessary for mitophagy and mitochondrial quality control. In this section, we will summarize recent literature exploring Miro's role as a facilitator of mitochondrial contact with other organelles.

Miro at ER-Mitochondria contact sites

Contact between the mitochondria and the ER was discovered over half a century ago.[14] The interaction of these organelles is of such frequency that contact sites can be physically isolated as mitochondria-associated membranes.[15] A robust body of research[16–20] has developed around the structure and function of this contact where both calcium and phospholipids are exchanged. Recent evidence indicates that Miro plays a critical role in facilitating and regulating this interaction.

Before discussing the regulation of ER-mitochondrial contact sites by Miro, we will first discuss the composition of this interaction. Many proteins localize to the area of contact between ER and mitochondria, forming a physical bridge (Fig. 2). This interaction is known as the ER-mitochondria encounter structure (ERMES) in yeast and as the ER-mitochondria contact site (ERMCS) in mammals.[21,22] The partners involved in the connection differ depending on the process being engaged. The less complex organism is perhaps a better-suited place to examine the basic functionality of the complex.

In yeast, Gem1p is an OMM protein within the Miro family that is responsible for mitochondrial inheritance and is required for regulation of the ERMES complex. Gem1p was first identified by its homology to human Miro and characterized as a factor recognizing cell signaling events to affect mitochondrial dynamics.[23] Deletion strains of *gem1* had difficulty growing on non-fermentable carbon source media and suffered from anomalous mitochondrial morphology and inheritance but maintained cristae organization. Its functional correlation to Miro was further demonstrated by mutations to disrupt GTPase activity, demonstrating their necessity for mitochondrial inheritance.[24] This was also the first study to demonstrate the GTPase activity of a Miro homolog *in vitro* and determined the impact of active site mutations on hydrolase activity.

Subsequent studies determined that Gem1p regulates the formation of the ERMES complex. This was verified by colocalization of a recombinant fluorescent Gem1p with another ERMES component at the ER-mitochondria interface in addition to mass-spectrometry-based approaches.[25,26] Additionally, in *gem1* deletion strains, there is reduced formation of the ERMES complex, indicating that Gem1p facilities ERMES formation. Further, mutations affecting GTP binding and hydrolysis decreased localization of exogenous fluorescently-labeled Gem1p to ERMES foci.[25] Mutation of the first EF-hand to impede calcium-binding mimicked the distribution of the hydrolysis mutant. Similar mutations in the cGTPase domain did not affect localization but failed to rescue the synthetic lethality of a deletion mutant deficient in phospholipid exchange. These experiments incorporated mutations in the second EF-hand as well, but the phenotypes were indistinguishable from wild-type and respective mutant backgrounds. This work establishes that the nGTPase and EF-hand 1 play important roles in ERMES complex formation, suggesting that allostery from these domains contributes to Gem1p function.

Gem1p's role in mitochondrial dynamics was expanded with tomography.[27] ERMES components and resident ER and mitochondria components were modified with fluorescent fusion proteins. Gem1p localized at contact sites where mitochondria were undergoing ER-associated mitochondrial division, in which the ER tubules form and constrict mitochondrial membranes, contributing to the creation of "daughter" mitochondria.[28] Once divided, the Gem1p proteins were found to be clustered at one tip after segregation. Taken together, the yeast version of Miro appears to be involved in directing the function of ERMES, instructing ER components to share membrane components, initiate fission, or perform other roles in ER-mitochondria interaction.

Mammalian Miro retains this role but has a broader array of potential regulatory targets in the ERMCS. ER-Outer mitochondrial membrane (OMM) partners include PS2-MFN2, BAP31-Fis1, IP3R-VDAC, VAPB-PTPIP51, and ORP5/8-PTPIP51[29] (Fig. 2). Some clearly function in biochemical regulation. For instance, the high concentration of calcium in the ER lumen is shuttled out through the inositol 1,4,5-trisphosphate receptor (IP3R)[30] and accepted by the OMM voltage-dependent anion-selective channel.[31] ORP5/8 facilitates the metabolism of phospholipids by exchanging lipid transfer proteins.[32,33] BAP31-Fis1[34] and PACS-2[35] have been implicated in pro-apoptotic pathways[36] while others have currently unelucidated functions.

Many studies have examined the components and functional linkage of ERMCS to regulatory pathways, but how and according to what signal they form has only recently come under scrutiny. The earliest observation of ERMCS disruption in a multicellular eukaryote came from an examination of neural stem cells from *Drosophila* bearing a protein-null mutant allele of Miro.[4] Homozygous crosses of *dMiro*^{B682} suffered greatly reduced brain size and lacked the Deadpan marker that accompanies brain development.[22] The authors manipulated protein levels using Gal4 induction to suppress or overexpress dMiro, manipulate other genes involved in calcium homeostasis for complementation experiments, and employed fluorescent probes for calcium ions to investigate the cause of the phenotype. They found that defects arising from alterations to dMiro expression could be partially or fully rescued with complements of ER-resident calcium channels and receptors. They further demonstrated that dMiro was specifically phosphorylated at conserved residue S66 (S59 in human) by Polo kinase and that this modification resulted in the accumulation of dMiro at the ER surface. Co-immunoprecipitation revealed that phospho-S66 dMiro associated with VDAC/Porin and Mitofusin/Marf, components of the ERMCS. Further, S66 phosphorylation enhanced the activity of the N-terminal GTPase domain, implying that the GTP hydrolysis activity of

Miro plays an active role in recruiting Miro to the ERMCS. Thus, these results indicate that Miro plays a critical role in supporting the formation of the ERMCS.

Lee et al. followed this study by investigating the effect of the Miro-ERMCS interaction on calcium flux in a Parkinson's disease model.[37] They examined dopaminergic neurons bearing mutations in PINK1, the kinase responsible for priming Miro for degradation or bearing transgenes for overexpression or knockdown of Miro. When Miro was upregulated, either by removal of PINK1 or Gal4 overexpression, the calcium concentration in mitochondria increased. This correlated with increased mitochondria size and eventual cell death. Removing Miro by knockdown attenuated the effect in the PINK1 mutant, bringing mitochondrial calcium levels close to wild-type. Further, knockdown of genes involved in calcium transfer within the ERMCS attenuated the calcium elevation from Miro overexpression, as did inhibition of IP3R and the mitochondrial calcium uniporter with small molecules. Likewise, knockdown of Polo rescued neuron loss from Miro overexpression, while overexpression of Polo exacerbated neuron loss except when Miro was knocked down or mutated to a phospho-null state (dMiro S66A). These results heavily support a role for Miro in directing calcium influx through the ERMCS into mitochondria.

Recently, a pair of heterozygous mutations in Miro were identified in a cohort of Parkinson's patients.[38] Patient fibroblasts bearing R272Q and R450C mutations were treated with thapsigargin, which depletes ER calcium into the cytosol. Control cells were able to re-establish normal cytosolic calcium levels, but calcium levels remained high in the mutant cells. Simultaneous blockade of the MCU resulted in elevated cytosolic calcium in both mutant and wild-type cells, implying a defect in mitochondrial calcium uptake or recycling to the ER. Imaging with tracking dyes revealed an overall decrease

in ERMCS, which in turn induced a reduction in the ER area and an increase in mitochondrial clearance by LC3-independent autophagy.

Miro's role in ERMCS was further characterized using super-resolution microscopy techniques.[39] Double knockout of Miro1 and Miro2 in mouse fibroblasts caused a decrease in ER-mitochondria overlap and distinct changes to mitochondrial morphology. They found that the absence of Miro causes a shift toward short, round mitochondria that partially lacked normal cristae architecture, while reintroduction returned morphology to longer, thinner mitochondria with normal, contiguous cristae. Immunoprecipitation of Miro identified interactions with components of the mitochondrial cristae organizing structure (MICOS). Microscopic analysis confirmed a positive correlation for co-localization of outer-membrane Miro and inner-membrane MICOS, with Miro forming clusters containing both Miro-1 and Miro-2. In contrast, double knockout of Miro caused the dispersal of MICOS components throughout the inner membrane, with occasional areas completely devoid of the subject species.

Miro's presence at the ERMCS and its apparent regulatory partners have been identified, but how Miro's recognition of its environment alters its function to regulate the activities involved remains unclear. Miro's calcium-binding EF-hands are almost certainly involved in environmental sensing, but how does the molecule react to this information to translate it into action? Are the GTPase domains involved at all? How does phosphorylation by Polo initiate the process? The molecular details may hold the keys to decipher the regulatory timing and decode its role in disease states.

Miro as a microtubule motor protein receptor for peroxisomes

Peroxisomes are "multipurpose organelles" whose contents are diverse, varying between organisms but also between tissues or cells of single organisms.[40] Their contents are

frequently toxic to the cell at large, consisting of metabolic enzymes required for redox equilibrium, reactive oxygen species neutralization, and bile acid synthesis, as well as signaling proteins modulating inflammation and innate immune signaling, among others.[41] Like mitochondria, peroxisomes carry critical functions that must be distributed throughout the cell. Indeed, peroxisomes can assist in regulating the redox environment around mitochondria, so the two organelles often need to be in proximity for proper function.

It is, therefore, not surprising that both peroxisomes and mitochondria utilize a similar long-range Miro-based transport mechanism. Miro has recently been identified as a component of peroxisomal trafficking.[42] Specifically, a splicing variant of Miro1 (named Miro1-var4) contains an insert between the C-terminal GTPase domain and membrane anchor that is recognized by the cytosolic receptor Pex19p for post-translational targeting to the peroxisomal membrane.[43] When the C-terminal membrane anchor was replaced with a peroxisome-targeted sequence, the resulting recombinant expression in COS-7 cells demonstrated differential peroxisome migration.[44] Recombinant peroxisome-targeted Miro bearing a constitutively active N-terminal GTPase domain caused accumulation of peroxisomes at the cell periphery, implying the activated GTPase adopts a preference for kinesin-driven movement. On the other hand, a peroxisome-targeted dominant-negative N-terminal GTPase mutant scattered the peroxisomes throughout the cytoplasm. Expression of the wild-type peroxisome-targeted construct in healthy and diseased patient-derived fibroblasts caused proliferation rather than the accumulation of peroxisomes. This points to a potential role in which Miro employs the force contributed by bound microtubule motors to exert pull forces that are likely to be at least partially responsible for organelle movement or dynamics.

The role of Miro in peroxisome transport has been supported by another recent study,[45] however, in this work Miro was implicated in peroxisome fission. In fibroblasts bearing

knockout mutations of Miro1, Miro2, and double knockouts, they observed the same number of long-range peroxisomal transport events as wild-type cells. However, in the Miro knockout cells, the authors observed a decreased peroxisomal size. From this, they conclude that Miro's primary role in the context of the peroxisome is to negatively regulate Drp1 recruitment, inhibiting fission. This indicates that Miro may play multiple roles on peroxisomal surfaces, facilitating transport or regulating fission, or both roles simultaneously. Further work will be needed to arrive at a coherent model of Miro's role in peroxisome dynamics.

The mechanism of microtubule attachment through Miro occurs via Pex1 and KIFC3[46] (Fig. 1), which is in contrast to axonal mitochondria that utilizes TRAK1 and KIF5B.[47] Interestingly, the retrograde transport machinery for Miro1-var4 is shared with mitochondria given that TRAK1, TRAK2, and dynein/dynactin are required for both peroxisome and mitochondrial transport.[48] Importantly, this process appears to be distinct from a hitch-hiking model of peroxisome transport, whereby peroxisomes are tethered to endosomes via Pex1 to facilitate transport.[49] Peroxisomes can also bind directly to microtubules via Pex14,[50] an essential component of peroxisome import machinery. Thus, these data indicate that peroxisomes utilize a similar mode of microtubule-based transport as mitochondria.

While it would be reasonable to assume that the mode of transport of the two organelles is similar, the molecular details have not been determined. The Miro variant study did not address whether the peroxisomal movement is arrested by calcium influx, like mitochondrial movement.[51] Also, there are several components in mitochondria implicated in motor adapter attachment, including mitofusin[52] and MICOS,[39] which are absent from peroxisomes. How similar these processes are and the extent to which Miro is involved remain open for study.

Miro coordinates mitochondrial transport on both microtubule and actin cytoskeleton

In order to maintain the health of neurons, mitochondria must be transported and positioned throughout neuronal bodies. As the required distances can reach up to one meter in length, the creation, transport, and positioning of mitochondria is a formidable task for the neuron.

The bi-directional transport of mitochondria along microtubules is well established, considering that the first evidence of mitochondrial transport occurred nearly 30 years ago.[53] Since its discovery, the key players and pieces of the regulatory mechanism have been characterized. As a C-terminally anchored OMM protein, Miro plays a key role as the receptor onto which microtubule motor proteins assemble[4,11] [Fig. 3(A)]. Miro cooperates with the adaptor protein Milton (*Drosophila*), also known as TRAK1 and TRAK2 in mammals, to recruit kinesin and dynein to drive anterograde and retrograde mitochondrial transport[3,11,47,48,54–56] [Fig. 3(B), 2(C)]. Based on mouse knockout studies, Miro1 plays a dominant role in both axonal and dendritic transport, as loss of Miro1 leads to a dramatic reduction in mitochondria motility.[57] As discussed below, even though Miro2 does play a role in connecting mitochondria to microtubules,[52] there are multiple pathways that connect mitochondria to microtubules considering that Miro1-Miro2 double knockout neurons still have axonal mitochondria (albeit with reduced velocity).[52]

Considering that modes of microtubule motor regulation are reviewed elsewhere,[56,58] here we will review some of the latest findings regarding mitochondrial movement patterns, actin-based transport, and Miro-independent mitochondrial movement.

Actin anchors stationary axonal mitochondria

The dynamic of the axonal mitochondrial transport changes as the neuron matures. In primary neuronal cultures, visualization of axonal mitochondrial transport indicates that

roughly one-third of mitochondria are motile, whereas two-thirds are stationary.[53,59–61] However, examining the axonal mitochondrial transport in vivo revealed that motility of mitochondria is gradually decreased with neuronal maturation, which coincides with increased localization to presynaptic sites and axonal boutons.[62–64] While previous work indicated that regulatory factors such as syntaphilin could anchor mitochondria in the presence of calcium,[65,66] there were very few tools available with which to probe this phenomenon.

In order to study this process, a new photocleavable small molecule was developed that could be used to artificially dimerize and then cleave two proteins in living cells.[67] This molecule - 'Zapalog' - induces a physical interaction between two target proteins by rapidly dimerizing recombinant fusions bearing FKBP and DHFR domains. Then, exposure to 405 nm wavelength radiation instantaneously photocleaves zapalog, causing rapid dissociation of the two proteins. Importantly, replenishing zapalog replaces the cleaved molecule to re-establish dimerization.

By exploiting these properties, the authors utilized zapalog to recruit a highly processive kinesin motor, KIF1a, to the mitochondrial surface in order to manipulate positioning. Consistent with the previous literature,[56] ~30% of mitochondria were motile and ~70% were stationary before adding zapalog. Adding zapalog caused the majority (60.3%) of mitochondria to rapidly switch to anterograde movement. After the photocleavage of zapalog, the behavior of the mitochondrial population returned to normal, indicating the endogenous mitochondrial motor complex retained its activity during the zapalog treatment.

A surprising result from this study was that 36% of axonal mitochondria remained stationary after inducing KIF1a recruitment via zapalog.[67] This work shows that

immovable mitochondria are mostly found at presynaptic sites. Depolymerizing actin filaments reduces the number of stationary mitochondria, indicating actin-based anchoring plays a role in tethering mitochondria at presynaptic sites.

This work expands the classification of mitochondrial movement status as either 'motile' or 'stationary' to three categories: motile, immotile but moveable, and immoveable. Immobilization of mitochondria at presynaptic sites ensures the stable production of ATP and calcium buffering capacity[68] to meet the local demands of synapses.

Myo19 - a new link between Miro and actin cytoskeleton

In addition to microtubule-based motility, actin-based mechanisms also play a role in the distribution of mitochondria inside cells. In budding yeast, the inheritance of mitochondria by the bud is mediated by the actin-based motor myosin-V.[69] In human neurons, actin-based motors may transport mitochondria to regions where microtubules are interrupted or may facilitate mitochondrial anchoring.[70]

Over ten years ago, Myo19 was implicated in actin-mediated mitochondrial movement and positioning in human cell lines.[71] Human Myo19 is a 970 amino acid length protein consisting of a myosin motor domain, a neck region, and a short tail that is widely expressed in multiple tissues and cell lines.[71] Immunostaining and truncation assays demonstrated that Myo19 localizes to mitochondria through its tail domain. Given that overexpression of full-length Myo19 leads to dramatic increases in the velocity of motile mitochondria, Myo19 acts as an actin-based motor that facilitates mitochondrial transport.[71]

After the discovery of Myo19, biochemical studies have started to shed light on its kinetic properties. Myo19 is a plus-end-directed,[72] actin-activated ATPase.[73] It is able to

translocate actin filaments in gliding assays.[72,73] Myo19 motor activity is necessary for mitochondrial motility, as Myo19 containing a mutation impairing ATPase activity did not alter mitochondrial network dynamics.[73]

The physiological function of Myo19 has been elusive since its discovery. Initial studies revealed that Myo19 connects mitochondria to actin and plays a role in controlling the distribution of mitochondria during cell division, thus ensuring their fair inheritance into the daughter cells.[74] Following this observation, additional work found that Myo19 facilitated the formation of starvation-induced filopodia by positioning mitochondria into the filopodia.[75,76]

How is Myo19 tethered to the mitochondria? Originally, Myo19 was thought to act as a peripheral membrane protein,[75] interacting with lipids directly. However, recent work discovered that Miro1/2 are receptors for recruiting and stabilizing Myo19 to the mitochondria based on knockout studies of Miro1 and Miro2, in addition to proximity labeling approaches[52,77,78] [Fig. 3(C), 3(D)]. Biochemical studies demonstrated that the Myo19 tail contains both Miro1/2-independent and Miro1/2-dependent mitochondrial targeting sites.[77,78] Interestingly, Miro1/2-mediated recruitment is regulated by the nucleotide state of Miro1/2 N-terminal GTPase domain.[77] Miro bearing a point mutation (T18N) in the N-terminal GTPase domain failed to recruit Myo19 tail to the mitochondria. These results suggest Miro1/2 plays a critical role in coordinating microtubule- and actin-based mitochondrial movements.

To summarize, Myo19 is a novel myosin that transports mitochondria along the actin filament. How it coordinates (or competes) with microtubule-based motors remains unclear. Given that Miro functions as a receptor for both TRAK1/2 and Myo19, further

studies are needed to understand how Miro preferentially interacts with actin vs. microtubule cytoskeleton.

Miro-independent transport of mitochondria along microtubules via Mitofusin-TRAK1/2

Recently, Miro-independent transport of mitochondria along microtubules was found in Miro knock-out cells for Miro1 and Miro2.[52] In this study, the authors explored the ability of mitochondria to undergo directed cytoskeletal transport in the absence of Miro. Considering that knocking out Miro1 in mice is lethal,[57] the authors derived mouse embryonic fibroblast cells in order to study mitochondrial transport. When monitoring mitochondrial transport in Miro1 and Miro2 knock-out cells, the authors noticed that motor proteins remained associated with mitochondria and that mitochondria underwent short-range transport. Specifically, kinesin-1 and dynein/dynactin motor proteins in addition to their TRAK1/2 adaptors remained associated with mitochondria in Miro knock-out cells.[52] Interestingly, even though long-range fast axonal transport was inhibited, mitochondria were distributed within axons, indicating that additional mechanisms can connect mitochondria to microtubules.[57] Furthermore, co-expression of TRAK1 or TRAK2 with kinesin-1 (KIF5C) still induced a dramatic redistribution of mitochondria to the cell periphery,[52] suggesting the existence of other TRAK1/2 receptors on the mitochondrial membrane.

How do motor proteins remain attached to mitochondria in Miro knock-out cells? Previous work indicated that mitofusins (Mfn1 and Mfn2) could be potential TRAK1/2 receptors given that they interact with Miro1/2 and TRAK1/2 in co-immunoprecipitation assays[79] and 3D-SIM super-resolution imaging analysis.[80] Mitofusins are dynamin family GTPases that mediate mitochondrial fusion by tethering opposing membranes[81,82] [Fig. 3(A)].

Mfn2 can regulate mitochondrial transport directly in neurons. Mitochondria exhibit decreased velocity and increased pausing time in Mfn2 knock-out neurons or neurons expressing mutated Mfn2,[79] suggesting that Mfn2 is required for mitochondrial transport. On the other side, TRAK1 has been shown to cooperate with Mitofusins to regulate mitochondrial fusion. The depletion of TRAK1 resulted in the fragmentation of mitochondria into small tubules and spheres, with decreased mitochondrial fusion rate[80]. Overexpression of TRAK1 caused the appearance of elongated and enlarged mitochondria, a sign of mitochondrial hyperfusion.[80] TRAK1 was hypothesized to promote mitochondrial fusion by tethering mitochondria together. In mitofusin knock-out cells, mitochondria appear to be fragmented with small globular structures.[80,83] Overexpression of TRAK1 results in extensive clustering of mitochondria in these cells.[80] Finally, electron microscopic analysis of mitochondrial structure revealed that the distance between the adjacent mitochondrial outer membrane is about 12 nm,[80] which is similar to the reported distance for Mfn1-mediated mitochondrial tethering.[82]

Mitochondrial transport requires acetylation of Miro

Recent work has highlighted a new dimension of Miro regulation by acetylation. In this study, the authors initially noticed that inhibition of HDAC6 (histone deacetylase 6) promoted axonal growth.[84] Upon closer examination, the authors determined that when HDAC6 deacetylated Miro, mitochondrial transport was inhibited. This indicated that Miro-directed mitochondrial transport requires acetylation. Proteomic studies of lysine acetylation previously indicated that sites K92, K512, and K616 are acetylated.[85] Expanding on this, Kalinski et al. demonstrated that lysine 105 is critical for transport, although the mode of transport regulation remains to be determined.

Mitochondrial motor protein 'shedding' during mitosis

During mitosis, mitochondria must be inherited equally between mother and daughter cells. To achieve this, mitochondria detach from microtubule motors in order to facilitate mitochondrial inheritance by the daughter cells.[86] Motor protein detachment occurs via phosphorylation of motor proteins by the kinases CDK1 and Aurora A. This detachment is critical considering that inappropriate tethering of mitochondria to microtubules during early stages of mitosis leads to binucleate cells and asymmetric mitochondrial inheritance.[86] Interestingly, during the later stages of mitosis, mitochondria regain microtubule attachment via Miro to Cenp-F in order to track the growing ends of microtubules for proper distribution into daughter cells.[13,87,88] Therefore, the regulation of microtubule attachment by Miro serves as a critical step in the proper inheritance of mitochondria to daughter cells.

Miro: Allosteric regulation with wide-spread cellular consequences

Cell biology-based investigation of Miro's GTP state indicates that specific nucleotide states are required for Miro's cellular activities. For example, when the nGTPase is GDP-bound, Miro facilitates Drp1-dependent mitochondrial fission.[89] However, when a similar mutation is studied within the context of microtubule-based transport, nGTPase in the GDP-bound form leads to a block in both microtubule and actin-based transport.[77,90] This effect on transport appears to be limited to the nGTPase as GDP-stabilizing or GTP-stabilizing mutations to the cGTPase have little to no effect on transport.[90] These results indicate that further work is needed to understand how Miro's GTP state connects to its diverse cellular activities.

Miro's GTPase activity has been best characterized in Gem1p, the yeast homolog of human Miro. In Gem1p, both of the GTPase domains were shown to hydrolyze GTP[24]. *Drosophila* Miro was also shown to have GTPase activity,[22] however, a full enzymatic

analysis was not performed on *Drosophila* Miro, making it difficult to compare with Gem1p. Recently, both the nGTPase and the cGTPase of human Miro1/Miro2 were shown to be catalytically active,[7] although this study did not investigate intact Miro enzymes nor did they characterize enzyme mechanisms. More detailed investigations into the catalytic activity of Miro by comparing the effect of calcium and binding to other cellular factors (e.g. TRAK1/2) will be necessary to draw a conclusion about the GTPase within the context of Miro activity.

Due to the putative GTPase activity of Miro, the role of GTPase-activating proteins (GAPs) and guanine-nucleotide exchange factors (GEFs) in regulating Miro activity remains an active area of research. A few potential GAPs/GEFs have been discovered recently, although much more research needs to be done to further characterize their role in Miro regulation. Interestingly, the only published GAP for Miro is VopE, a *Vibrio cholera* Type III secretion system effector.[91] During infection, VopE stimulated mitochondrial redistribution, whereby instead of mitochondria clustering around the nucleus they were transported to the cell periphery via kinesin. The authors proposed that VopE acted as a GAP for Miro based on the observed interaction of VopE with Miro and the increased Miro GTPase activity when VopE was added. It remains to be determined whether there is a native GAP for Miro.

To date, two potential GEFs for Miro have been identified, GBF1 and Vimar.[89,92] For GBF1, a physical interaction between GBF1/GTP-bound Arf1 and Miro was demonstrated.[93–95] The combination of mitochondrial phenotype changes and the physical interaction between GBF1 and Miro demonstrates that GBF1 could act as a GEF for Miro.[92] In *Drosophila*, Vimar was identified to partially localize to the mitochondria and physically interact with/act as a GEF for Miro.[89] For characterizing the GTPase activity of

Miro, a GAP or GEF may be the missing piece that assists Miro with the hydrolysis of GTP and subsequent replacement with unhydrolyzed GTP.

Miro: Calcium regulation

Beyond its GTPase activity, Miro possesses tandem EF-hands allowing Miro to bind to Ca^{2+}. EF-hands consist of a helix-loop-helix motif that binds to calcium[96] [Fig. 1(C)]. There is extensive evidence to suggest that the EF-hands of Miro act as calcium-dependent molecular switches to regulate mitochondrial transport in axons and dendrites in mammalian cell lines. This is supported by live-cell imaging studies indicating that the influx of cytoplasmic Ca^{2+} paused mitochondrial transport.[12,51] Moreover, when the EF-hands of Miro were mutated to E208K and E328K to block Ca^{2+} binding, mitochondrial transport was no longer inhibited by Ca^{2+}.[51]

While there is agreement on the inhibition of mitochondrial transport via Ca^{2+}, there are competing models for the molecular mechanism. Three distinct models have emerged related to kinesin-mediated inhibition: 1) dissociation of kinesin (KIF5B) from Miro-TRAK complexes;[12] 2) allosteric control of motor protein activity;[51] 3) recruitment of an anchoring factor (syntaphilin).[66] Using *in vitro* translated proteins, Macaskill *et al.* saw that the addition of Ca^{2+} led kinesin to separate from Miro-TRAK complexes.[12] In contrast, cell-based transfection studies by Wang and Schwarz observed that Miro-TRAK-kinesin complexes remained intact, but instead lost microtubule-binding abilities due to the binding of kinesin to Miro directly.[51] Finally, using a combination of cell-based transfection pull-downs and reconstituted recombinant proteins, Chen and Sheng demonstrated that kinesin binds to syntaphilin directly.[66] These competing models indicate that further work is needed in order to provide decisive insight into how Ca^{2+} inhibits transport.

Finally, beyond the transport of mitochondria, the shape of mitochondria is also regulated by Ca^{2+}. A recent study links Miro's calcium-binding capacity to mitochondrial shape transition that occurs to differentiate mitochondria between long networked organelles that are more efficient at energy production and shorter, round organelles that are frequently associated with pathological states or apoptotic signals.[99] Live-cell imaging revealed that mitochondrial shape change occurred prior to the opening of the permeability transition pore, mitochondrial swelling, and calcium uptake through the mitochondrial calcium uniporter. Likewise, shape transition was independent of Drp1/dynamin-dependent fission machinery. Mutations made to Miro-1 and Miro-2's EF-hands confirmed that EF-hand 1 of Miro1 was the calcium-sensing determinant for shape transition, and the process did not require Miro's GTPase activity.

In addition to directly binding Ca^{2+}, Miro interacts with the mitochondrial calcium uniporter, MCU, which has been shown to regulate Ca^{2+} influx into the mitochondria.[97] While the N-terminus of MCU is not required for mitochondrial targeting, it is essential for binding to Miro-1.[98] Calcium elevation triggers proteolytic cleavage of MCU, which disrupts the interactions between MCU and Miro1, thereby reducing calcium influx and mitochondrial movement.[97,98] This coupling between MCU and Miro1 suggests that there may be an interplay between Ca^{2+} influx and mitochondrial motility.

Considering that mitochondria buffer cytosolic Ca^{2+} levels and Miro's ability to interact with MCU, Miro may be able to sustain or limit the influence of Ca^{2+} on mitochondrial processes. This line of thinking suggests that if Miro alters MCU activity, Miro may promote longer periods of high cytosolic Ca^{2+} levels, thus altering mitochondrial biology (inhibiting transport, mitochondrial shape). Further work will be needed to determine how Miro may be able to regulate Ca^{2+} levels directly through its interaction with MCU.

Miro regulation in neurodegenerative disease

As a critical component of axonal mitochondrial transport, Miro occupies a central role in regulating mitochondrial position and turnover, a process critical to the health of neurons.[100] In this section, we will discuss the link between Miro and mitochondrial biology with neurodegeneration.

Accumulating evidence is providing a link between proper mitochondrial function and dendritic tree development, a distinct process from axonal transport. These observations come from studying Miro1 knockout mouse animal models, where the deletion of Miro1 resulted in reduced mitochondria within dendrites.[101] This, in turn, reduced the size and complexity of dendrites, indicating that Miro1 is critical for supporting dendritic health.[101] The importance of Miro1 for dendrites was further underscored by conditional knockouts, where Miro1 was deleted from mature neurons. In these cells, loss of Miro1 leads to loss of dendritic mitochondria and dendritic complexity, ultimately leading to neurodegeneration.[101] Considering that axonal mitochondrial distribution was unaffected in Miro1 knockout cells, the dramatic loss of dendritic complexity in Miro1 knockout cells mirrors evidence from other forms of neurodegeneration where dendritic complexity is reduced.[102] Indeed, in a mouse model for Alzheimer's disease, loss of dendritic complexity was linked to changes in the electrical properties of the neurons.[103] This all indicates that, since mitochondria are critical for supporting dendritic complexity, Miro may occupy a central role in many forms of neurodegeneration.

While proper mitochondrial function may be critical in many forms of neurodegenerative disease, we will focus on one such disease - Parkinson's disease - as there is a growing body of work linking Miro and mitochondrial clearance to Parkinson's disease.

Parkinson's disease

Parkinson's disease is a neurodegenerative disorder that causes the progressive loss of dopaminergic neurons, leading to impaired motor function associated with α-synuclein aggregates (Lewy bodies).[104] Affecting nearly 680,000 individuals in North America as of 2010,[105] Parkinson's disease represents a growing malady with an estimated impact of 10 million individuals worldwide.[106] While there are many different aspects of cell biology that are impaired in Parkinson's disease, we will focus this review on the role of mitochondrial turnover and the impact that Miro has on this process.

Over the past 20 years, studies of human patient mutations in combination with cell biological studies have produced a model of mitochondrial surveillance via PINK1 (kinase: PTEN-induced kinase 1) and Parkin (E3 ubiquitin ligase) that is critical to support healthy neurons.[107] In healthy mitochondria, PINK1 is translated into the OMM, at which point it is translocated into mitochondria for eventual proteolytic degradation. This indicates that PINK1 levels are typically low in healthy mitochondria. During mitochondrial damage such as membrane depolarization, PINK1 is no longer degraded and instead resides as a membrane-anchored component in the OMM. In this new localization, PINK1-mediated phosphorylation of Parkin leads to Parkin activation. Once activated, Parkin-mediated ubiquitination signals the initiation of mitophagy, which is the selective degradation of mitochondria by the autophagosome.

While human genetics, cell biology, and biochemical studies have implicated PINK1 and Parkin as critical components necessary for stimulating mitochondrial turnover, the mechanism connecting these proteins to mitophagy remained poorly understood until recently. A big breakthrough occurred nearly 10 years ago with the identification of PINK1 as a subunit of a multi-subunit complex comprising Miro and TRAK1.[108] Subsequent work identified Miro as a target of both PINK1 phosphorylation and Parkin ubiquitination in

order to degrade damaged mitochondria.[109] Moreover, recent studies suggest that Parkin directly associates with Miro prior to activation by PINK1,[110] suggesting that Miro has direct physical connections to both Parkin and PINK1. These initial results suggested that dissociating mitochondria from the microtubule network is a requirement for the successful turnover of mitophagy.

Phosphorylation & ubiquitination of Miro is required for mitophagy

Miro phosphorylation regulates mitophagy, serving to promote or inhibit mitochondrial turnover. PINK1 phosphorylates Miro1 residues S156, T298, and T299[109,111] [Fig. 1(A)]. Probing the role of these phosphorylation sites through phosphomimetic mutants, S156E effectively recruited Parkin although this was insufficient to drive mitophagy. However, mimicking phosphorylation of Miro at T298/T299 inhibited the ubiquitination of Miro, the recruitment of Parkin, and the Parkin-dependent arrest of mitochondria.[111] This indicates that the specific phosphorylation status of Miro may serve as a cellular signal regulating mitophagy.

After phosphorylation by PINK1, Miro is ubiquitinated by Parkin.[112] In mouse embryonic fibroblasts, mitochondrial damage resulted in Miro ubiquitination on multiple lysine residues [Fig. 1(A)].[112] Preventing the ubiquitination of Miro stabilized the level of Miro after mitochondrial damage and slowed mitophagy.[112] Interestingly, the ubiquitination kinetics between mutant K572R and a Miro mutant where all lysines were mutated to arginines were not significantly different.[112] These results suggest that K572 plays a critical role in the ubiquitination of Miro, however, other residues may still play a role. While K572 seems to be fundamental to the ubiquitination of Miro1, more work is needed to determine the necessity of the other ubiquitination sites for the regulation of Miro and subsequent effects on mitophagy.

Recently, these results connecting targeted Miro degradation in order to promote mitophagy have been further supported through studies with Parkinson's disease-derived patient samples. First, by comparing induced pluripotent stem cells (iPSCs) from both healthy and Parkinson's disease patients, Wang and coworkers extended the initial work of PINK1 and Parkin to include leucine-rich repeat kinase 2 (LRRK2).[113] Specifically, the authors compared mitochondrial motility after mitochondrial stress in LRRK2-mutation backgrounds, sporadic Parkinson's disease, and healthy individuals to demonstrate that while healthy iPSC-derived neurons showed slowing and degradation of mitochondria following mitochondrial stress, both LRRK2 and sporadic Parkinsons' disease neurons did not have any change in mitochondrial motility or turnover. Beyond the role of LRRK2, parallel investigations of Parkinson's disease patient samples demonstrated that increased α-synuclein levels are associated with increased Miro expression and mitochondrial motility where α-synuclein may be directly interacting with Miro.[114]

Based on these recent findings, a more detailed model of PINK1 and Parkin-mediated mitophagy is emerging, placing Miro at the center of the process (Fig. 4). Under normal conditions, prior to mitochondrial damage, Miro directs microtubule-based transport via kinesin and dynein. In this condition, Parkin is associated with Miro but is not activated. Upon damage, PINK1 accumulates on the OMM but is unable to phosphorylate Miro until LRRK2 phosphorylates Miro. After Miro is phosphorylated by LRRK2, PINK1 phosphorylates both Miro and Parkin to activate Parkin's activity as an E3-ubiquitin ligase. Once activated, Parkin ubiquitinates Miro, thus targeting Miro for proteasomal degradation and subsequent mitochondrial clearance via mitophagy.

Given that this process is modified by changes to the regulatory factors LRRK2, PINK1, and Parkin (Fig. 4), there may be therapeutic strategies to increase the amount of mitophagy. In Parkinson's disease patient-derived neurons, impairment of flux through

this pathway resulted in decreased mitochondrial clearance via mitophagy. Indeed, there are increased levels of mitochondria in addition to higher levels of Miro expression.[115] Based on these observations, an *in silico* small molecule inhibitor of Miro was developed with the goal of reducing Miro levels in order to promote mitophagy.[115] Promisingly, 'Miro reducer' decreased Miro expression levels in Parkinson's disease neurons and increased mitophagy, potentially opening the door to future therapeutic strategies to promote the clearance of damaged mitochondria from Parkinson's disease patients.

Future directions and questions

Over the past 20 years, there has been significant progress in our understanding of Miro's role in the regulation of mitochondrial cell biology. We believe this review has demonstrated that Miro sits at the center of a number of mitochondrial biology processes: organelle contact sites, mitochondrial transport, and mitophagy. By sitting at this nexus, Miro may act a lynchpin to allow cellular processes to have multiple rippling effects; through changes in Miro, the cell can simultaneously redistribute mitochondria (via microtubules, shape transitions), degrade old mitochondria, or alter other organelle processes. This aspect of Miro is both the most exciting and the most daunting to study. Future work will require a systems-level understanding of cellular physiological in order to unpack the pleiotropic nature of Miro within mitochondrial biology.

We are excited for the next twenty years as we believe this field will be ushered into an era of mechanistic understanding of mitochondrial biology. During this time, we hope that the following questions will help to guide future cell biological, biochemical, and structural understandings of Miro with the goal of developing a deep understanding that can be translated into the clinical setting:

- How does Miro interact with myriad binding partners and how is binding preference determined?

- Does the GTP state of Miro regulate the context through which Miro acts on cellular processes?
- How does Ca^{2+} inhibit mitochondrial motility?
- What is the stoichiometry of Miro's interaction with binding partners?
- How does PINK1/LRRK2/Parkin uncouple Miro from microtubules to promote mitophagy?

We believe that continued advances in our cell biological and biochemical understanding of Miro will help to characterize how a single protein is able to sit at the center of many critical cellular processes.

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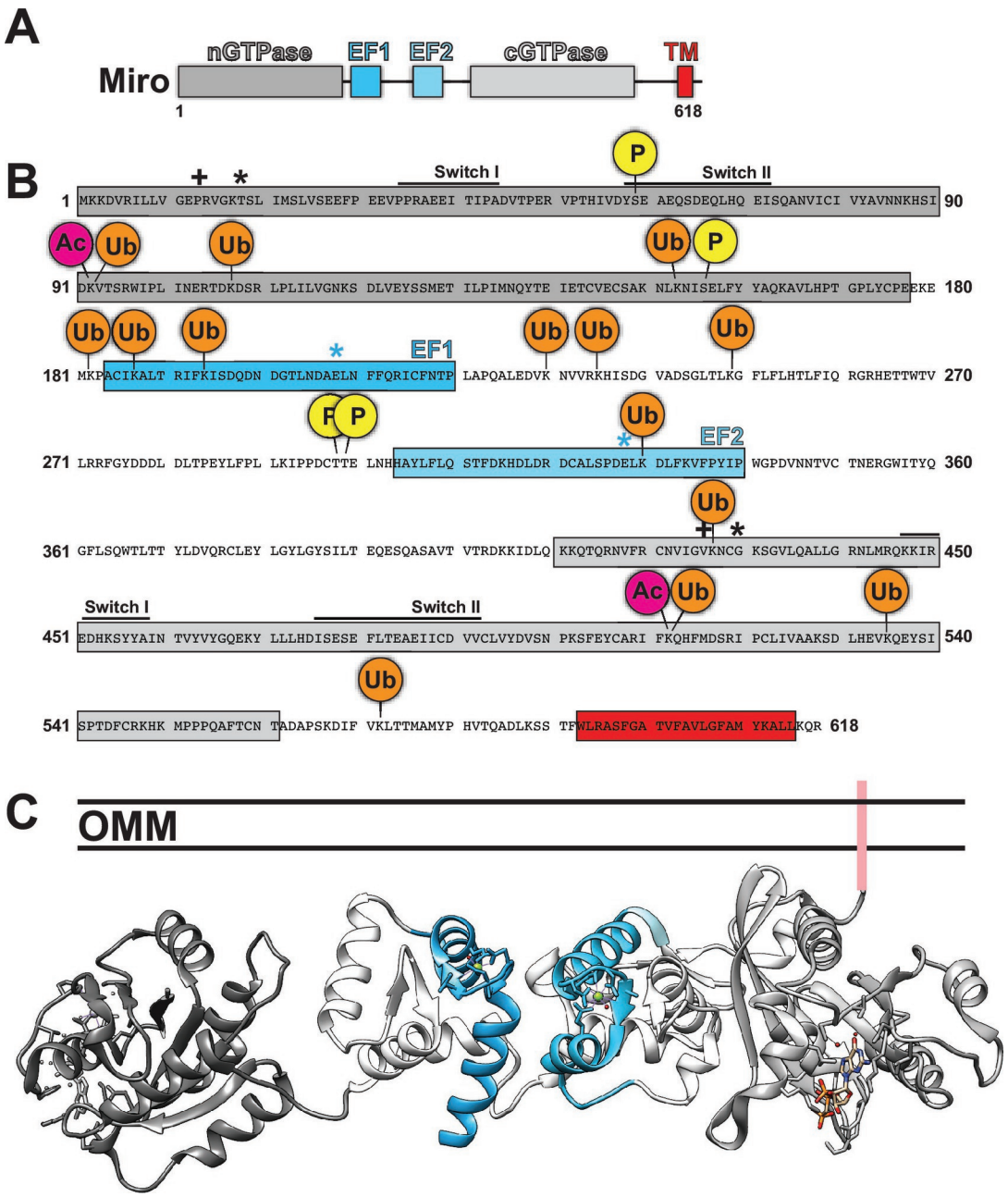
Figure legends

Figure 1. Structural features of Miro. (A) Domain organization. (B) Annotated primary sequence of Miro. Black '+' indicates constitutively active GTP mutant whereas '*' indicates constitutively inactive GTP mutant. Blue '*' indicate mutations in EF-hands that block calcium binding. (C) Model of Miro structure based on published coordinates of Miro's nGTPase (PDB: 6D71) and the C-terminus of Miro (PDB: 5KSZ).

Figure 2. Miro mediates organelle-cytoskeleton and organelle-organelle contact sites. Miro contributes to diverse cellular activities involved in organelle-cytoskeleton contacts (Peroxisome-microtubule, Mitochondria-microtubule, & Mitochondria-actin) as well as organelle-organelle contacts (ER-Mitochondria & Mitochondria-Mitochondria).

Figure 3. Miro-directed transport of mitochondria (A) Receptors localized on outer mitochondrial membrane. (B) Regulatory factors that influence transport on microtubules and actin. (C) Motor protein machinery required for transport. (D) Example receptor-motor complexes docked onto mitochondria & cytoskeletal elements.

Figure 4. Potential model of mitochondrial clearance via mitophagy. Under normal cellular conditions, Miro directs microtubule-based transport via kinesin and dynein. Note that Miro is also pre-bound to Parkin. (1) Mitochondrial damage (e.g. membrane depolarization) occurs. (2) Following damage, LRRK2 is activated and phosphorylates Miro. (3) Once phosphorylated by LRRK2, PINK1 phosphorylates Miro in addition to Parkin. (4) Parkin ubiquitinates Miro. (5) Mitophagy clears damaged mitochondria. Note that mutations to LRRK2, PINK1, and Parkin (denoted '*') in addition to α-synuclein accumulation likely inhibits this process to prevent mitochondrial clearance.



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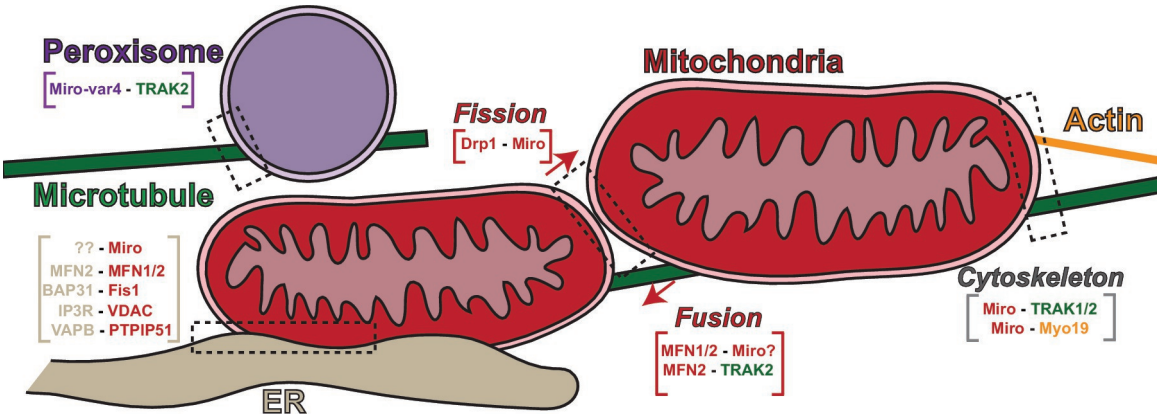

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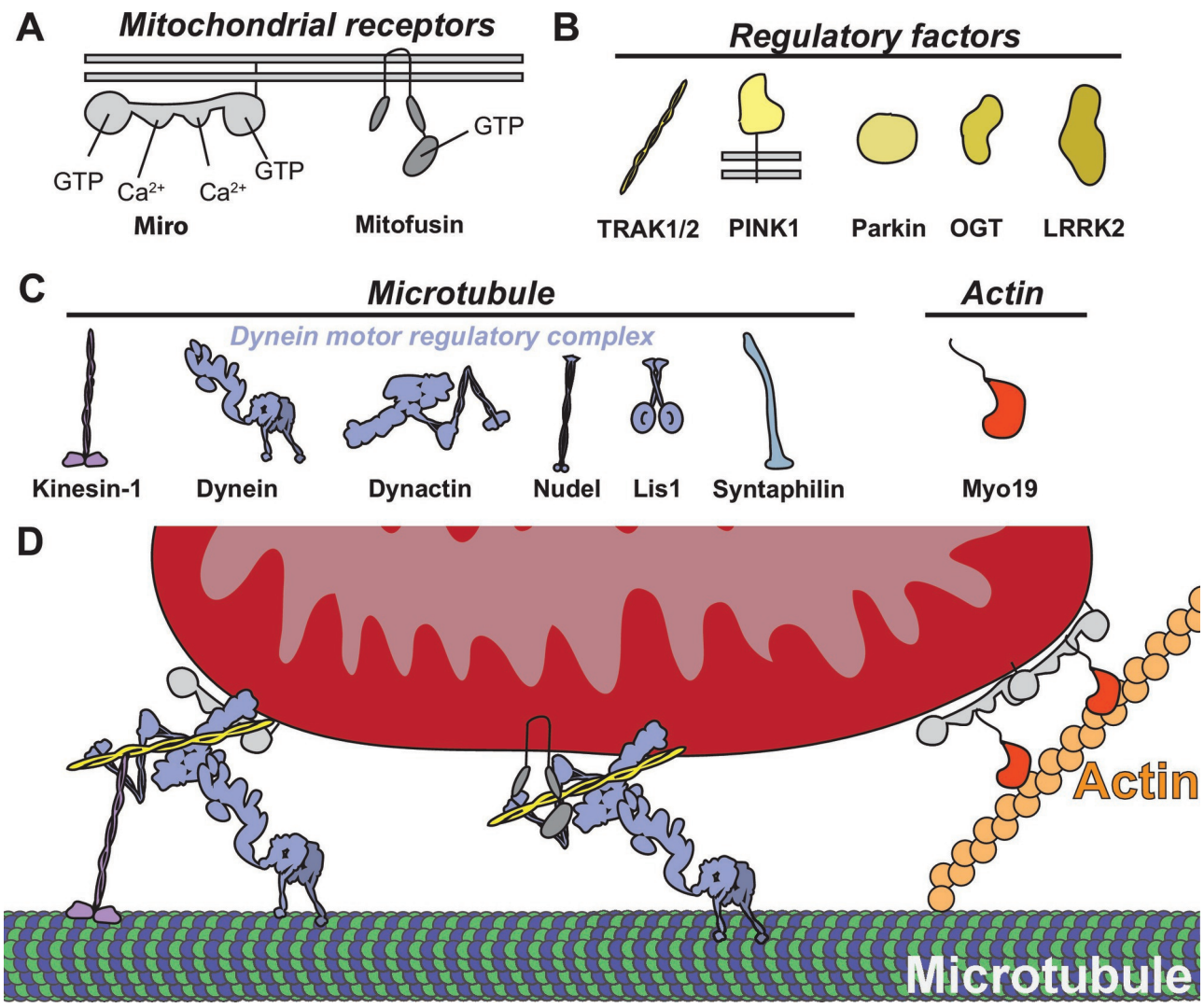

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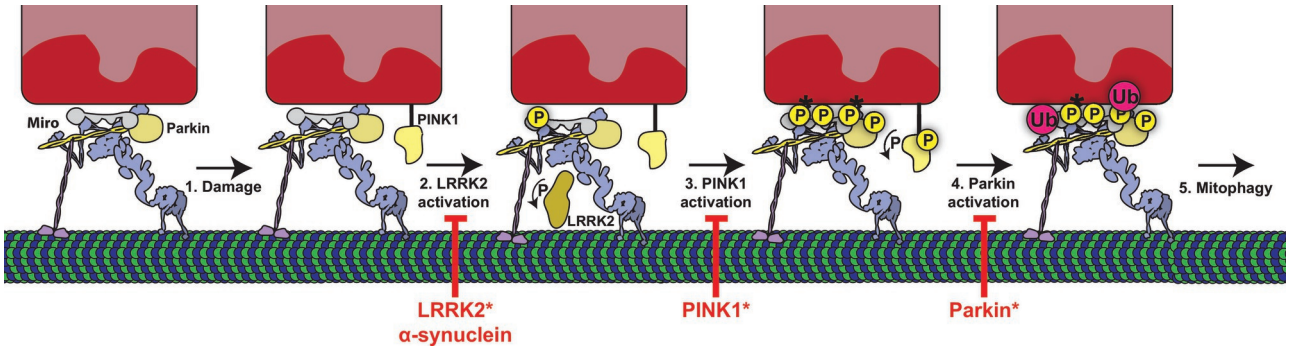

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