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Trak1 is a novel regulator of mitochondrial fusion and cellular homeostasis

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B.S., University of Rochester, 2007

Advisor: Lian Li, Ph.D.

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Abstract

Trak1 is a novel regulator of mitochondrial fusion and cellular homeostasis

By: Crystal Anne Lee

Trafficking protein, kinesin-binding 1 (Trak1) is a ubiquitously expressed protein functioning in two separate cellular pathways, mitochondrial transport and endosome-to-lysosome trafficking. Trak1 mutations are linked to several disease states. In mice, a homozygous frameshift mutation in the *Trak1* gene causes a recessively transmitted form of hypertonia. In humans, elevated levels of Trak1 protein expression have been reported in several types of cancers and a Trak1 variant is linked to childhood absence epilepsy. However, the pathogenic mechanisms of Trak1 in these diseases states remain unknown. In my dissertation work, I identified a new function for Trak1 as a regulator of mitochondrial fusion independent of its role in mitochondrial transport and I found Trak1 to be essential to mitochondrial health and cell survival under cellular stress conditions. Furthermore, I found that the hypertonia-associated mutation of Trak1 exhibits partially disrupted targeting to mitochondria, suggesting a novel mechanism of hypertonia pathogenesis. In addition, I show that the *Drosophila melanogaster* orthologue of Trak1, Milton, is essential for viability and identified a functional role for Milton in the endocytic pathway, establishing the conserved dual functions of Trak1 and Milton. Together the work described in this dissertation, reveals a novel function for Trak1 in mitochondrial fusion and cellular homeostasis, and demonstrates that Trak1 is critical to both cellular and organismal health.

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Introduction and Background

1.1. Opening remarks

Mutations in trafficking protein, kinesin-binding 1 (Trak1) protein have been linked to several disease states, including hypertonia, childhood absence epilepsy, and cancer (An et al., 2011; Chioza et al., 2009; Gilbert et al., 2006; Zhang et al., 2009). The molecular and cellular pathogenic mechanisms of Trak1 mutations are unknown. Trak1 is a highly conserved and ubiquitously expressed protein. The localization of Trak1 to both early endosomes and mitochondria, as well as the reported roles of Trak1 in endocytic trafficking and mitochondrial transport suggest that Trak1 functions in at least two separate cellular pathways (Brickley and Stephenson, 2011; Webber et al., 2008). A better understanding of the functional role of Trak1 will lead to critical insights into the underlying disease mechanisms and how Trak1 dysfunction may lead to a wide range of disease states, as well as provide possible therapeutic targets for these diseases.

1.2. Trak1 protein

Human trafficking protein, kinesin-binding 1 (Trak1) protein is composed of 953 amino acids, contains three coiled-coil domains (Figure 1-1), and is mapped to chromosome 3p22.1 (Iyer et al., 2003). The human Trak1 gene contains 35 exons and is predicted to produce 10 transcripts or splice variants, although no other splice variant other than the 953 amino acid isoform has been described in the literature. Trak1 is ubiquitously expressed in all tissues, including brain, heart, skeletal muscle, lungs, spleen, smooth muscle, liver, kidney, and testis (Gilbert et al., 2006; Iyer et al., 2003). Trak1 is also widely detected throughout the central nervous system (CNS), including the cortex, cerebellum, hippocampus, thalamus, spinal cord, the forebrain, and the midbrain (Gilbert et al., 2006) and widely detected throughout development (van Spronsen et al., 2013). Trak1 is evolutionarily conserved from flies to humans and has been reported to be essential for survival in *Drosophila melanogaster*, as Trak1 null mutants die as first instar larvae (Glater et al., 2006). No Trak1 homologs have been reported in the genomes of

nematodes, plants, or yeast, suggesting that Trak1 function may not be required in lower life forms.

Trak1 was initially identified in 2003 as a binding partner and substrate for the enzyme β-O-linked N-acetyl glucosamine (O-GlcNAc) transferase (OGT) (Iyer et al., 2003). For this reason Trak1 is also known as OIP106 for OGT-interacting partner of 106 kDa. The C-terminal residues 639-859 of Trak1 are sufficient to directly bind OGT and include the sites of Trak1 glycosylation by OGT (Iyer and Hart, 2003). Post-translational modification of nuclear and cytoplasmic proteins by OGT results in addition of O-GlcNAc monosaccharides to serine and threonine residues and acts as a regulatory modification similar to phosphorylation. The OGT/Trak1 interaction is conserved from flies to mammals, and a recent study suggests that OGT modification of Trak1 is dynamically regulated by glucose levels, suggesting Trak1 function may be sensitive to cellular glucose concentrations (Pekkurnaz et al., 2014).

The subcellular localization of Trak1 was first reported exclusively in the nucleus and absent from the cytoplasm, however, subsequent studies have shown Trak1 localization to be cytoplasmic, specifically to the early endosome and mitochondria, and absent from the nucleus. In 2003 Iyer et al. first documented endogenous Trak1 localization to the nucleus of HeLa cells (Iyer et al., 2003). They showed that endogenous Trak1 immunoreactivity was detected exclusively in the nuclear fraction and not the cytosolic fraction, and that endogenous Trak1 co-localized with RNA polymerase II in the nucleus by immunofluorescence confocal microscopy, and similarly by electron microcopy (EM), where gold particles against Trak1 where exclusively detected in the nucleus. However, studies using exogenously expressed human Trak1 reported exclusion of these constructs from the nucleus, and rather colocalization with mitochondria and early endosomes (Brickley et al., 2005; Fransson et al., 2006; Webber et al., 2008). Additionally, ectopic expression of Trak1 was shown to alter the distribution of mitochondria leading to the appearance of aggregated and thread-like mitochondria, but had no effect on endosome morphology or distribution. In 2006, Gilbert et al. generated a second Trak1 antibody and

reported immunoreactivity of endogenous Trak1 in the cytosol and neurites, while Trak1 was markedly absent in the nucleus of mouse brain tissue sections (Gilbert et al., 2006). In 2008, our lab generated a third Trak1 antibody and confirmed Gilbert's results that endogenous Trak1 was exclusively cytosolic, reporting colocalization of endogenous Trak1 with markers for the early endosome and mitochondria, but not with lysosomes or ER markers (Webber et al., 2008). The localization of endogenous Trak1 with mitochondria has also been reported in mouse brain and spinal cord sections using a fourth Trak1 antibody (van Spronsen et al., 2013). This group reported 80% overlap of endogenous Trak1 with the mitochondrial marker cytochrome c but did not examine colocalization with endosomes or other organelles. Van Spronsen et al. reported Trak1 detection in the neuronal cell bodies, axons, and dendrites; however, stronger Trak1 signal was detected in axons compared to dendrites establishing a polarized distribution of Trak1 favoring axons.

The first antibody generated against Trak1, by Iyer et al. used a peptide sequence corresponding to amino acids 1-20 of human Trak1, while those of Gilbert et al. used a sequence corresponding to amino acids 105-298, our lab (Webber et al., 2008) a sequence corresponding to amino acids 935-953, and van Spronsen et al. a sequence corresponding to amino acids 754-953, suggesting that the first antibody may be detecting an alternative spliced isoform or a proteolytically processed form of Trak1 that is not the predominant cytosolically localized 953-amino acid isoform.

Our lab first identified Trak1 as a novel regulator of endosome-to-lysosome trafficking (Webber et al., 2008). Endosomal sorting of internalized cell surface receptors to lysosomes for degradation is crucial in the control of cell signaling. Immunofluorescence confocal microscopy indicated that a population of endogenous Trak1 colocalizes with hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) on EEA1-positive early endosomes. Hrs is a protein component of the endocytic trafficking machinery known as Endosomal Sorting Complexes Required for Transport (ESCRTs), which is required for protein sorting from the early endosome

to lysosomes (Lauwers et al., 2009; Raiborg et al., 2002; Raiborg and Stenmark, 2009). Endogenous Trak1 was shown to interact with endogenous Hrs by coimmunoprecipitation (Webber et al., 2008). Evidence for direct binding between Trak1 and Hrs was demonstrated though *in vitro* binding assays with recombinant Hrs and Trak1. Additionally, a Trak1 deletion construct missing the predicted Hrs binding region (resides 359-507) was not capable of binding Hrs and did not localize to early endosomes (Webber et al., 2008). Epidermal growth factor (EGF) receptor endocytic trafficking assays, which are widely used to study trafficking of endocytosed cell surface receptors from the early endosome to lysosomes, showed that both the over-expression as well as the small-interfering-RNA (siRNA) mediated knockdown of Trak1 inhibited the degradation of internalized EGF receptors by blocking endosome-to-lysosome trafficking. This inhibitory effect of Trak1 over-expression on EGF receptor degradation is dependent on association of Trak1 with Hrs, as the Trak1 deletion construct missing the Hrs binding domain had no effect on EGF receptor trafficking. It is well established that both the over-expression and knockdown of Hrs inhibits EGF receptor degradation (Chin et al., 2001; Kanazawa et al., 2003; Stern et al., 2007), and because Trak1 has an identical effect, which requires the Hrs binding domain, this suggests that Trak1 acts though Hrs-mediated endosomal sorting.

Trak1 interacts with the $GABA_A$ receptor and a mutation in mouse Trak1 linked to hypertonia has been shown to reduce expression levels of $GABA_A$ receptors in the CNS (Gilbert et al., 2006), however it is not known how Trak1 regulates $GABA_A$ receptor expression. $GABA_A$ receptors are hetero-pentameric proteins comprised of combinations of the subunits α1-6, β1-3, γ1-3, δ, ε1-6, θ, and π depending on the developmental stage and localization in the central nervous system (Stephenson, 1995; Stephenson, 2006). The major $GABA_A$ receptor subtype in the adult brain is comprised of two $\alpha_{1/2}$, $\beta_{1/2}$, and one subunit of γ_2 (Stephenson, 1995). $GABA_A$ receptors are fast-acting, ligand-gated chloride channels that are the primary inhibitory neurotransmitter receptor in the CNS. A spontaneous homozygous truncation mutation in mouse

Trak1 (Trak1 hyrt) causes hypertonia, a neurological dysfunction resulting in tremor, jerky movements, and a hunched posture (Gilbert et al., 2006). Mice with homozygous Trak1 hyrt mutation have decreased levels of $GABA_A$ receptors, most notably in the spinal cord and brain stem, where an 80% decrease compared to wild-type mice was reported (Gilbert et al., 2006). The authors propose that Trak1 may regulate the endocytic trafficking of $GABA_A$ receptors to block their lysosomal degradation or to facilitate the trafficking of newly synthesized $GABA_A$ receptors to the cell surface but this has not been experimental shown.

A population of endogenous Trak1 is also localized to mitochondria. The role of Trak1 in mitochondrial transport was first elucidated in *Drosophila*, in which the fly homolog of Trak1 is called Milton. Milton was shown to function as an adaptor protein linking mitochondria by its association to Miro transmembrane Rho GTPases residing on the outer mitochondrial membrane (OMM) to kinesin heavy chain (KHC) to facilitate the transport of mitochondria on microtubule filaments in neuronal axons (Stowers et al., 2002). The function for Trak1 in mitochondrial transport has also been shown and like Milton, Trak1 functions as an adaptor protein linking mitochondria to microtubule filaments to facilitate their transport (Brickley et al., 2011; Brickley and Stephenson, 2011). The association of Trak1 with KHC is direct and requires the second coiled-coil region encompassing amino acids 201-306 (Smith et al., 2006; van Spronsen et al., 2013). In mammals, there are two Miro proteins (Miro1 and Miro2) and both have been found to coimmunoprecipitate with Trak1 (Fransson et al., 2006; Glater et al., 2006; Ogawa et al., 2014; Pekkurnaz et al., 2014; Wang and Schwarz, 2009). The Miro/Trak1/Kinesin mitochondrial transport complex is particularly critical in neurons. The highly polarized structure of neurons requires that mitochondria be transported from the cell body along axons to synapses, which can be several centimeters away (MacAskill and Kittler, 2010; Schwarz, 2013). Knockdown of endogenous Trak1 by RNA interference (RNAi) gene silencing in hippocampal neurons prevented the formation of the Miro/Trak1/Kinesin complex and consequently inhibited mitochondrial motility (Brickley and Stephenson, 2011). Trak1 gene silencing by small hairpin

RNA (shRNA) reduced mitochondrial motility in both the retrograde and anterograde directions and in both axons and dendrites, with a stronger reduction of moving mitochondria in axons (van Spronsen et al., 2013). Interestingly, although endogenous Trak1 is detected in the neuronal cell body, axons, and dendrites, Trak1 distribution is biased towards axons (van Spronsen et al., 2013). Additionally, transfection of GFP-tagged Trak1 showed biased targeting to axons and shifted mitochondrial localization to axons (van Spronsen et al., 2013). The dysfunction in mitochondrial transport due to depletion of Trak1 also resulted in abnormal axonal outgrowth and morphology: reduced axon length, the number of axonal tips, and the number of axon branches by 50% compared to control neurons (van Spronsen et al., 2013). The ability of Trak1 knockdown to inhibit retrograde mitochondrial transport suggests that Trak1 may also interact with dynein motors. Immunoprecipitation and mass spectrometry studies found Trak1 to interact specifically with the $p150^{Glued}$ subunit of the dynactin (van Spronsen et al., 2013). Dynactin $p150^{Glued}$ binds to dynein motors to facilitate retrograde transport. Trak1 contains two $p150^{Glued}$ binding regions; one mapped to the N-terminal domain requiring amino acids 1-360 and a second binding region at the C-terminus (amino acid residues 396-953) (van Spronsen et al., 2013).

1.2.1. Trak1 is part of the HAPN-domain family of proteins that also includes GRIF-1 and HAP1

In mammals, Trak1 has a homologue called GRIF-1. GRIF-1 shares 48% homology with Trak1 but has restricted expression to excitable tissues including the brain, heart, and skeletal muscle (Kirk et al., 2006; van Spronsen et al., 2013). In mice, GRIF-1 is expressed throughout the developing and adult brain, particularly in the cerebellum, cortex, and midbrain but is largely absent in the spinal cord, an axon-rich tissue where Trak1 is abundant (van Spronsen et al., 2013). In neurons, GRIF-1 displays punctate staining and co-localizes with mitochondrial markers as well as with vesicular structures including endosomes (MacAskill et al., 2009a). In rat pheochromocytoma PC12 cells, GRIF-1 was found localized with Hrs at early endosomes (Kirk et al., 2006). The N-terminus of GRIF-1 and Trak1 also share homology with Huntingtin-

associated protein 1 (HAP1) and collectively, these proteins are termed the HAP1 N-terminal homologous (HAPN) domain family of proteins (Figure 1-1) (Kirk et al., 2006; Webber et al., 2008). These proteins are structurally similar, containing three coiled-coil domains with the first two N-terminal coiled-coil domains encompassing the HAPN domain and have all been shown to interact directly with Hrs and function in endosome-to-lysosome trafficking of EGF receptors (Kirk et al., 2006; Li et al., 2002; Webber et al., 2008) (Table 1-1). Additionally, all three members have been shown to directly interact with subunits of the $GABA_A$ receptor and regulate its endosome-to-lysosome trafficking (Beck et al., 2002; Gilbert et al., 2006; Kittler et al., 2004). HAP1 has also been implicated in the endocytic trafficking of Trk A receptors (Rong et al., 2006), and the type 1 inositol (1,4,5)-trisphosphate (IP31) receptor (Tang et al., 2003). GRIF-1 has also been implicated in the trafficking of the potassium channel Kir2.1 to enhance Kir2.1 cell surface expression but does not alter the overall cellular levels of Kir2.1 (Grishin et al., 2006). Taken together, this family of proteins may have a general role in the regulation of endosome-to-lysosome sorting of a variety of cell surface receptors.

GRIF-1, which stands for $GABA_A$ receptor interacting factor-1, is also known as Trak2 and OIP98. GRIF-1 interacts with a wide variety of protein binding partners and was initially identified as a binding partner for the β2 subunit of the $GABA_A$ receptor (Beck et al., 2002), and independently identified as a binding partner and substrate for OGT enzyme (Iyer et al., 2003) and as a Hrs binding partner through a yeast two-hybrid screen (Kirk et al., 2006). GRIF-1 also directly binds to kinesin heavy chain (Brickley et al., 2005; Brickley and Stephenson, 2011) and to mitochondrial outer membrane proteins Miro 1 and 2 (Fransson et al., 2006; MacAskill et al., 2009a). Like Trak1, both the over-expression and knockdown of GRIF-1 was shown to inhibit the degradation of internalized EGF receptors and blocks their trafficking from early endosomes to lysosomes (Kirk et al., 2006). GRIF-1 directly binds Hrs, requiring the amino acid residues 359-507, and endogenous GRIF-1 colocalizes with Hrs on EEA1 positive early endosomes in NGF-differentiated PC12 cells (Kirk et al., 2006). The over-expression of Hrs recruits GRIF-1 to Hrs-

positive endosomes (MacAskill et al., 2009a), while the over-expression of GRIF-1 causes the clustering of EEA1-positive early endosomes in the perinuclear region (Kirk et al., 2006); this effect is dependent on the kinesin-binding region of GRIF-1, suggesting that GRIF-1 may regulate the microtubule transport of early endosomes. Thus GRIF-1 and possibly Trak1 may act as an adaptor linking Hrs containing endosomes to kinesin.

Similarly to Trak1, GRIF-1 also functions in mitochondrial transport. The exogenous expression of GRIF-1 in HEK293 cells results in rearrangement of mitochondria into large perinuclear aggregates (Brickley et al., 2005) and the exogenous expression of GRIF-1 in COS7 cells induces tubular and threadlike mitochondria (Fransson et al., 2006; Smith et al., 2006) as well as enlarged mitochondria (Koutsopoulos et al., 2010). The over-expression of Miro1 in neurons recruits GRIF-1 to mitochondria and enhances transport of mitochondria to distal neuronal processes (MacAskill et al., 2009a).

In neurons, Trak1 and GRIF-1 have been shown to have different contributions in the regulation of mitochondrial transport. Knockdown of GRIF-1 by gene silencing had no effect on mitochondrial motility in axons of hippocampal neurons but over-expression of GRIF-1 could rescue mitochondrial transport in Trak1 knockdown neurons (Brickley and Stephenson, 2011). Recently, it was shown that GRIF-1 is required for mitochondrial transport specifically in dendrites, while Trak1 is required for axonal mitochondrial transport (Loss and Stephenson, 2015; van Spronsen et al., 2013). This difference in neuronal localization is suggested to be through the utilization of different molecular motors as GRIF-1 predominately interacts with dynein/dynactin, while Trak1 interacts with Kinesin-1 and dynein/dynactin (van Spronsen et al., 2013). Antibodies against endogenous GRIF-1 protein label the neuronal cell body, axon, and dendrites, with more prominent localization in dendrites. GFP-tagged GRIF-1 was almost exclusively targeted to dendrites and shifted the mitochondrial localization to dendrites. Depletion of GRIF-1 by siRNA did not affect the motility of mitochondria in axons (Brickley and Stephenson, 2011; van Spronsen et al., 2013) but decreased the number of motile mitochondria in

dendrites, in both the anterograde and retrograde directions (van Spronsen et al., 2013). The ability of GRIF-1 to influence transport of mitochondria in both directions may be explained by the fact that in dendrites, microtubules are oriented in an antiparallel fashion and therefore, mitochondrial transport in both the anterograde and retrograde directions only requires dynein motors (Kapitein et al., 2010a; Kapitein et al., 2010b). The knockdown of GRIF-1 in developing neurons resulted in a decrease in dendritic length by 50% suggesting that GRIF-1 is required for the normal development of dendrites (van Spronsen et al., 2013). Although Trak1 and GRIF-1 are highly similar in their amino acid sequences and bind a similar set of proteins at related domains, it has been reported that *in vivo* GRIF-1 exists in a folded confirmation, in which the N-terminus and C-terminus interact through head-to-tail binding, while Trak1 does not (van Spronsen et al., 2013). This finding could possibility account for differences in Kinesin-1 binding affinities and differential localization in neurons. Furthermore, GRIF-1 expression is restricted to the brain, heart, and skeletal muscles while Trak1 expression is ubiquitous, suggesting that GRIF-1 may provide more specialized functions in tissues that require a greater level of regulation.

The autosomal dominant, progressive neurodegenerative disorder Huntington's disease (HD) is caused by expansion of a polyglutamine (poly-Q) tract in the huntingtin (htt) protein (Rubinsztein et al., 1993). Although htt is widely expressed in the brain and body, HD pathology is restricted to the brain, and the expansion of the poly-Q repeats is thought to result in a toxic gain of function through pathological interactions with other proteins (Gunawardena and Goldstein, 2005; Rong et al., 2006; Rubinsztein et al., 1993; Tang et al., 2003). HAP1 was the first protein identified to bind htt and the htt mutation enhances htt-HAP1 binding, sequestering HAP1, which may prevent its normal function (Li et al., 1995). HAP1 protein is primarily expressed in neurons and has a critical role in neuronal function (Gutekunst et al., 1998), suggesting that HAP1 dysfunction may contribute to the specific brain pathology of HD. The subcellular localization of HAP1 was shown to be cytoplasmic and also distributed to many types of membranous organelles, including mitochondria, endoplasmic reticulum, lysosomes, and

synaptic vesicles (Gutekunst et al., 1998). Several studies have implicated HAP1 in microtubule-based transport as HAP1 binds to kinesin light chain (KLC) (McGuire et al., 2006), the KIF5 kinesin motor proteins (Twelvetrees et al., 2010), and the $p150^{Glued}$ subunit of dynactin (Li et al., 1998). HAP1 has also been shown to be necessary for htt-mediated transport of brain-derived neurotrophic factor (BDNF)-containing vesicles along microtubules and mutant htt impairs this transport (Gauthier et al., 2004).

Like other HAPN domain family members, HAP1 also directly binds Hrs and regulates the endosome-to-lysosome trafficking of EGF receptors (Li et al., 2002). The over-expression of HAP1 induces enlargement of early endosomes. HAP1 was also found to interact with the β1 subunit of the $GABA_A$ receptor. The interaction of HAP1 with $GABA_A$ receptors inhibits their lysosomal degradation, leading to increased recycling; therefore, over-expression of HAP1 increases $GABA_A$ receptor activity (Kittler et al., 2004). Although the loss of HAP1 function is believed to contribute to HD pathogenesis, HAP1 gene knockout studies in mice have challenged this model, as studies in HAP1 knockout mice suggest that HAP1 may not be required for normal function of the adult brain. HAP1 knockout mice die shortly after birth and display depressed feeding behavior that results in death (Chan et al., 2002; Li et al., 2003); however, reduction of litter size to decrease competition for milk allowed a fraction of HAP1 null mutants to survive into adulthood (Dragatsis et al., 2004). These data suggest that HAP1 has an essential role for feeding in the early postnatal period but is not essential in adult mice; once HAP1 null mice survive into adulthood, they have normal feeding behavior, normal life spans, and no neurodegeneration, neuroanatomical, or behavioral defects were observed (Dragatsis et al., 2004). However, a prior report found degeneration selectively in the hypothalamus of HAP1 null mice, concomitant with degenerating mitochondria, and condensed and fragmented nuclei (Li et al., 2003). Furthermore, this group also reported reduced expression of EGF receptors in the cortex of HAP1 null mice. Although it is not known what accounts for the discrepancies reported in the

literature, one possibility is that the hypothalamic degeneration and reduced EGF receptor expression reported by Li et al. was due to starvation rather than directly to knockdown of HAP1.

1.2.2. Milton, the Drosophila homolog of Trak1

In *Drosophila melanogaster* there is single HAPN domain containing protein called Milton; therefore, Milton is effectively the *Drosophila* orthologue of mammalian Trak1, GRIF-1, and HAP1 (Figure 1-1). Milton protein shares 33%, 28%, and 25% overall identity to human Trak1, GRIF1, and HAP1 proteins, respectively. Milton was first identified in a *Drosophila* genetic screen for mutations that induce synaptic transmission defects (Stowers et al., 2002). Since mutations affecting axonal and synaptic function could be lethal at embryonic or larval stages, screens were carried out in mosaic flies that were homozygous for a mutant allele in only the eyes while the rest of the body was heterozygous. Flies with null mutations of Milton were blind due to loss of nerve excitation in the eye (Stowers et al., 2002). Furthermore, mitochondria were absent in terminals and axons, although mitochondria were abundant in the cell bodies. The transport of synaptic vesicles to terminals and axons were not affected, and the general architecture of the axons and synapses appeared normal, thus the transport defect was selective for mitochondria (Stowers et al., 2002). The loss of synaptic transmission in Milton mutant flies is likely due to the loss of mitochondria and the consequent decrease in ATP supply.

Exogenous expression of Milton in HEK293T cells resulted in rearrangement of the mitochondrial network into large aggregates and Milton colocalized with the mitochondrial marker MitoTracker Red (Stowers et al., 2002). Additionally, coimmunoprecipitation and mass spectrometry analyses revealed association of Milton with kinesin heavy chain (KHC) and the mitochondrial Rho GTPase Miro (Glater et al., 2006). Milton is proposed to function as an adaptor protein linking mitochondria to microtubule motors, accounting for the absence of

mitochondria in synaptic terminals of Milton null flies and the ability to rearrange mitochondria when over-expressed in cell culture.

Milton null flies do not attain the third larval instar, typically surviving only 3-5 days after hatching. Four different Milton transcripts, including mRNAs of 3.7, 4.0, 4.2 and 5.2 kb, can be detected by Northern blot analysis (Stowers et al., 2002). The Milton transcripts are expressed throughout development and are detected from early embryogenesis (as early as 0 – 2 hours after egg laying) to adulthood, with the highest transcript levels in the heads of adult flies, suggesting enrichment in neurons. There is a single *Milton* gene; however, at least four Milton transcripts are produced through alternative splicing, Milton A-D (Glater et al., 2006; Stowers et al., 2002). The Milton isoforms differ in their NH_2 termini due to different 5' ends, but their mRNA transcripts converge on exon 9, resulting in all 4 transcripts sharing amino acids 129-1116 of the Milton A isoform (Glater et al., 2006; Stowers et al., 2002). The widely studied Milton-A protein is composed of 1116 amino acids with a predicted molecular weight of 120 kDa; however, immunoblots of *Drosophila* head extracts using a Milton specific antibody raised against Milton A amino acid residues 908-1055 produces four bands ranging from 120-160 kDa, representing the four distinct Milton isoforms. Milton contains no recognizable structural motifs, except for 3 coiled-coil domains and all the isoforms retain these coiled-coil domains. The functional differences between the isoforms are not known. Each of the four isoforms localizes to mitochondria, however, only Miltons A, B, and D associate with KHC, while Milton C does not (Glater et al., 2006). An interesting possibility is that the alternative splicing of Milton could help differentially regulate the transport of mitochondria in flies through different binding affinities for KHC. One possibility is that Milton-C inhibits recruitment of KHC to keep a pool of mitochondria immobilized in the cell body. Another possibility is that there are tissue-specific splice variants in order to regulate mitochondrial transport according to the needs of particular cell types. The Milton-C splice variant may also be more similar in function to GRIF-1, which also binds poorly to kinesin. A third possibility is that the Milton splice variants may have

different functional contributions that in higher eukaryotes are carried out by the HAPN domain family of proteins individually.

Mapping studies indicate that Milton 1-450 was sufficient to coimmunoprecipitate KHC. Transfection of Milton 1-450 in COS7 cells revealed colocalization with KHC in aggregates, but not on mitochondria, while Milton 750-1116 did localize to mitochondria; thus the association of Milton to KHC and mitochondria are in separate domains (Glater et al., 2006). The region of KHC responsible for Milton association has been mapped to the KHC tail region 810-891 (Glater et al., 2006). The targeting of Milton to mitochondria is believed to require binding to Miro, as Milton does not contain a transmembrane domain or a mitochondrial targeting sequence. Yeast two hybrid screens detected an interaction between Milton and Miro (Giot et al., 2003) and Miro null flies also lack axonal mitochondria (Guo et al., 2005). Furthermore, Milton could coimmunoprecipitate Miro, and Miro lacking a transmembrane domain could displace Milton from mitochondria when cotransfected into COS7 cells (Glater et al., 2006). Therefore, Miro is likely responsible for recruitment of Milton to mitochondria by serving as a receptor for Milton on the outer mitochondria membrane. In eukaryotic cells Milton/Trak1 is required for kinesin mediated mitochondrial transport on microtubule tracks, however, in yeast which lack a Milton/Trak1 homolog, mitochondria motility is actin based using myosin motors and requires the yeast Miro homolog, GEM1p. GEM1p mutants have abnormal mitochondria distribution, suggesting that in less complex organisms Miro alone can regulate mitochondrial transport (Frederick et al., 2004).

1.3. Endocytic trafficking and regulation

The endocytic pathway is critical for cell survival, development, and receptor mediated signaling. At the plasma membrane, cell surface receptors and other transmembrane proteins are internalized either constitutively, in response to ligand binding, or induced by post-translational modifications such as phosphorylation and ubiquitination (Katzmann et al., 2002) (Figure 1-2).

Once internalized, these cell surface proteins are targeted to the early endosome where a sorting decision is made. At the early endosome, endocytosed membrane proteins are either recycled back to the cell surface or sorted to intraluminal vesicles of multivesicular bodies (MVBs) for transport to lysosomes for degradation (Katzmann et al., 2002; Maxfield and McGraw, 2004). The recycling pathway is believed to be constitutive and occurs as the default pathway (Verges et al., 1999), while targeting to the lysosome is signal mediated, requiring ubiquitination of the cytoplasmic domain of endocytosed membrane proteins (Barriere et al., 2007).

Ubiquitin is a small peptide composed of 76 amino acids that is covalently linked to lysine residues on the target protein through an enzymatic cascade that includes an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme, and an E3 ubiquitin ligase (Pickart, 2001). Although the conjugation of ubiquitin to proteins regulates a broad range of cellular functions, the number of ubiquitin moieties as well as the type of ubiquitin linkage directs proteins to different cellular fates. For example, proteins marked with a polymer of ubiquitins (a polyubiquitin chain) through K48-linkage selectively targets proteins to the 26-S proteasome for degradation, whereas the addition of a single ubiquitin moiety (mono-ubiquitination) has been shown to be sufficient to promote endocytosis (Hicke, 2001). Sorting complexes on endosome membranes contain ubiquitin-interacting domains. One of these sorting proteins is Hrs, which is the first protein recruited to early endosomes and becomes enriched in regions of the endosome containing a specialized bilayer of clathrin (Raiborg et al., 2002; Raiborg et al., 2001; Sachse et al., 2002). Using immunoelectron microscopy, lysosomal-targeted receptors such as epidermal growth factor (EGF) receptors were found concentrated in the clathrin coated areas but not receptors set to be recycled back to the plasma membrane such as transferrin receptors (Sachse et al., 2002), providing a role of the endosomal clathrin coat in protein sorting towards the lysosome pathway.

Hrs is proposed to be involved in the retention of ubiquitinated cargo proteins within the bilayer clathrin coat and has been shown to interact with both mono-ubiquitin (Sachse et al.,

2002) and K63-linked poly-ubiquitin chains on cargo receptors (Barriere et al., 2007; Huang et al., 2006). Hrs is also responsible for recruitment of the Endosomal Sorting Complexes Required for Transport (ESCRT) I, II, and III. The ESCRTs are responsible for incorporating the cargo proteins into vesicles that bud into the lumen of multivesicular bodies (MVBs) (Lauwers et al., 2009; Raiborg et al., 2002), which eventually fuse with lysosomes, where the cargo are then degraded by lysosomal hydrolases. The ESCRT complexes sequentially recruit one another from the cytoplasm to the surface of the endosome and have well defined subdivision of labor. Specifically, components of ESCRT-0 (Hrs, STAM), ESCRT-I (Tsg101, Vps28, Vps37, Mvb12), and ESCRT-II (EAP45, EAP30, EAP20) contain ubiquitin-binding domains (UBD) and recognize the endosome-enriched phospholipid, phosphatidylinositol 3-phosphate (PtdIns(3)P) at the early endosome surface for cargo recognition and sorting (Henne et al., 2013; Raiborg and Stenmark, 2009; Roxrud et al., 2010). ESCRT-II initiates assembly of ESCRT-III complex (CHMPs, Alix, IST1), which is a membrane-remodeling machine that causes inward vesiculation of endosomal membranes to generate the internal vesicles of MVBs (Raiborg and Stenmark, 2009). The ESCRT-III machinery also recruits deubiquitinating enzymes to recycle the ubiquitin molecules from the ubiquitin-tagged MVB cargo for reuse (Babst et al., 2002). The release of ESCRTs from the endosome membrane is attributed to the AAA ATPase VPS4 complex so that ESCRTs are recycled for additional cargo sorting events and to terminate the MVB pathway (Babst et al., 1997; Babst et al., 1998).

Hrs is an evolutionarily conserved protein that initiates endosomal protein sorting. Hrs is ubiquitously expressed and contains a phosphatidylinositol 3-phosphate-binding FYVE domain that confers endosomal targeting (Raiborg et al., 2001). Hrs was originally identified as a tyrosine phosphorylated protein in melanoma cells stimulated with hepatocyte growth factor (Komada and Kitamura, 1995). The tyrosine phosphorylation of Hrs occurs when cells are treated with hepatocyte growth factor, epidermal growth factor, and platelet-derived growth factor, as well as some cytokines including interleukin-2 and granulocyte-macrophage colony stimulating factor

(Asao et al., 1997; Komada and Kitamura, 1995). Furthermore, the phosphorylation of Hrs requires internalization of cargo receptors such as receptor tyrosine kinases (RTKs) (Urbe et al., 2000) as well as an intact ubiquitin-interacting motif (UIM) in Hrs (Urbe et al., 2003). Mouse embryos with a homozygous null mutation of the Hrs gene have defects in ventral folding morphogenesis, resulting in development of the embryonic ventral region outside of the yolk sac, as well as cardiac bifida, lack of a foregut, and death at embryonic day 11 (Komada and Soriano, 1999). Significantly enlarged early endosomes were detected in cells from Hrs null embryos, similar with observations seen in yeast mutants lacking Vps27p, the homolog of mammalian Hrs, which also exhibit enlarged endosomes and accumulation of endocytosed proteins at early endosomes (Komada and Soriano, 1999; Piper et al., 1995). The over-expression of Hrs has been shown to inhibit the trafficking of cargo proteins from the early endosome to late endosomes/lysosomes but not interfere with endocytosis or recycling (Chin et al., 2001; Raiborg et al., 2001). The over-expression of Hrs leads to accumulation of EGF receptors in Hrs positive endosomes. In *Drosophila*, embryonic Hrs mutant cells display more EGF receptor signaling activity and accumulate activated receptors and ubiquitinated proteins in enlarged endosomes (Jekely and Rorth, 2003; Lloyd et al., 2002). In *Drosophila*, accumulation of other types of signaling receptors, including PDGF/VEGF receptors, Patched, Smoothend, Notch, Thickveins, are also seen in Hrs mutant cells (Jekely and Rorth, 2003). Depletion of other ESCRT components have also been shown to inhibit formation of MVBs, cause endosomal enlargement, and block endosome-to-lysosome transport (Babst et al., 2000; Bache et al., 2004; Doyotte et al., 2005; Kanazawa et al., 2003). Mutations in ESCRT components have also been linked to tumorigenesis and neurodegenerative diseases (Stuffers et al., 2009).

1.3.1. Consequences of endosomal-lysosomal pathway dysfunction

The endocytic down-regulation of numerous signaling receptors, including EGF receptors, are critical in modulating the extent and rate of the signaling pathways they activate and thus, are critical to the maintenance of normal cellular homoeostasis. Once receptors are internalized and trafficked to endosomes, attenuation of receptor signaling can be achieved by facilitating their degradation through the MVB-lysosome pathway. Dysfunction in this pathway is implicated in neurodegenerative disease and cancer pathogenesis (Saksena and Emr, 2009). Receptor tyrosine kinase (RTK) signaling pathways regulate a wide range of biological processes, including cell growth, proliferation, migration, and survival. The excessive signaling of EGF receptors, a very well studied RTK, for example, is associated with the development of several types of cancers in humans (Boeckx et al., 2013; Hirsch et al., 2013; Misale et al., 2014; Smith et al., 2015). There is a growing body of evidence that dysfunction in ESCRT components can lead to tumorigenesis, since ESCRTs mediate the sorting of cargo receptors into the MVB-lysosome pathway. Failure to degrade signaling receptors can result in enhanced receptor recycling back to the plasma membrane, or accumulation of activated receptors on endosomes where they can continue to hyper-activate downstream signaling events leading to the increased tumorigenic potential of the cell. The ESCRT-1 components Tsg101 and Vps37A are considered to function as tumor-suppressor genes and are often deleted or mutated in cancers (Li and Cohen, 1996; Xu et al., 2003).

Neurons are particularly vulnerable to defects in the endosome-lysosome pathway and defects in this pathway have been linked to a number of neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), HD, and amyotrophic lateral sclerosis (ALS) (Bahr and Bendiske, 2002; Nixon, 2005; Nixon et al., 2008). Mutations in *alsin*, the GEF (guanine-nucleotide-exchange factor) responsible for the endosomal fusion regulator Rab5, has been shown to cause ALS (Yang et al., 2001). Abnormal ubiquitin-positive protein deposits are found in affected neurons of ALS patients. Mutations in CHMP2B, an ESCRT-III component,

have been observed in some patients with ALS (Parkinson et al., 2006). A splice mutation in CHMP2B causes a rare form of autosomal dominant frontotemporal dementia (FTD) in a large Danish pedigree (Skibinski et al., 2005), providing a direct link between dysfunction in endosomal sorting machinery and neurodegeneration. FTD is the second most common form of presenile dementia after AD and is characterized by the presence of tau and ubiquitin-positive inclusions (Talbot and Ansorge, 2006). Additionally, spastin, a protein mutant in hereditary spastic paraplegia interacts directly with the ESCRT-III component CHMP1B (Reid et al., 2005). Furthermore, the over-expression and knockdown of ESCRT components have been shown to affect neuronal cell survival. For example, the over-expression of Alix/AIP1, an ESCRT-II complex subunit, induced cell death in cerebellar neurons (Mahul-Mellier et al., 2006; Trioulier et al., 2004). In mice, knockout of STAM1, an ESCRT-0 complex subunit, results in extensive neuronal cell death in the hippocampus and cerebral cortex (Yamada et al., 2001). The ESCRT-I subunit Tsg101 was shown to be a substrate of the E3 ubiquitin ligase Mahogunin, and null mutations of Mahogunin cause spongiform neurodegeneration in mice (Kim et al., 2007a). Spongiform neurodegeneration, best known as the hallmark of prion disease, is characterized by vacuolation in neurons and extensive neuronal death. Depletion of Mahogunin results in enlargement of early endosomes and inhibition of EGF receptor trafficking from the early endosome to lysosomes. The finding that Mahogunin regulates endosome-to-lysosome trafficking, possibly through Mahogunin-mediated ubiquination of Tsg101, suggests that dysregulation of endocytic trafficking contributes to the pathogenic mechanism of spongiform neurodegeneration.

Neurodegenerative diseases often are characterized by accumulation of intracellular ubiquitinated protein aggregates and one possibility is that inhibition of the degradative MVB pathway seen in ESCRT mutants lead not only to inappropriate receptor signaling but also intracellular accumulation of aberrant proteins. Additionally, the lysosome system is also responsible for degradation of long-lived proteins and organelles though the macroautophagy pathway. Macroautophagy, also known simply as autophagy, is a highly conserved process from

yeast to humans that is responsible for the clearance of proteins and organelles by engulfing them in a double-membraned vesicle called the autophagosome, which fuses with lysosomes for degradation of autophagic contents (Klionsky and Ohsumi, 1999). Although classically it has been assumed that the endocytic pathway and the autophagic pathway converge terminally at lysosomes, autophagosomes have been found to fuse with endosomal compartments (Berg et al., 1998; Tooze et al., 1990) and efficient autophagy requires functional MVBs, suggesting that defects in the endosomal-lysosomal pathway also inhibit autophagy. Defective autophagy leads to accumulation of misfolded protein aggregates in the cytosol. It has been shown that disruption of the autophagy pathway can itself cause neurodegeneration even in the absence of disease associated mutant proteins (Hara et al., 2006; Komatsu et al., 2006), suggesting that the continuous clearance of cellular proteins through autophagy prevents their pathological accumulation and subsequent neuronal dysfunction and neurodegeneration. A protective role for autophagy has been implicated in preventing neurodegeneration (Rubinsztein et al., 2007). In HD, for example, the induction of autophagy enhances the clearance and ameliorates the toxicity of mutant huntingtin in cell, *Drosophila*, and mouse models (Ravikumar et al., 2002; Ravikumar et al., 2008; Ravikumar et al., 2004). The induction of autophagy can also clear the A53T mutant form of α-synuclein that causes inherited PD (Berger et al., 2006; Webb et al., 2003). The most important risk factor for the development of neurodegenerative diseases is aging (Collier et al., 2011; Hung et al., 2010) and the gradual loss of lysosomal degradation capacity occurs during the normal aging process (Bahr and Bendiske, 2002; Cuervo and Dice, 2000; Nakamura et al., 1989), suggesting that lysosomal dysfunction may be a major contributing factor in age-related neurodegenerative disorders. Moreover, the experimental induction of lysosomal dysfunction can recapitulate some important pathological features of age related-neurodegeneration (Hajimohammadreza et al., 1994; Okada et al., 1994; Takauchi and Miyoshi, 1989). Therefore, the proper regulation of protein degradation whether through the endosomal-lysosomal pathway, autophagy, or other protein degradation pathways are critical in maintaining cellular health.

1.4. Mitochondria

In eukaryotes, mitochondria are essential organelles that are responsible for the majority of ATP energy production, calcium buffering, and the regulation of apoptotic cell death. Mitochondria form an interconnected network that is constantly undergoing fusion, fission, transport, and mitophagy; these processes are collectively termed mitochondrial dynamics (Chen and Chan, 2009; Detmer and Chan, 2007b) (Figure 1-3). Mitochondrial morphology is controlled by the precise regulation of fusion and fission cycles. These opposing processes work together to maintain the shape, size, and number of mitochondria, as well as enable content mixing between mitochondria to keep a homogenous and healthy mitochondrial population. Mitochondria have two membranes that act in a concerted fashion for proper fusion and fission to occur, adding to the complexity in the regulation of these processes (Fritz et al., 2001; Loson et al., 2013; Meeusen et al., 2004; Smirnova et al., 2001). Mitochondrial transport primarily occurs along microtubules to move mitochondria to areas where energy is needed or where calcium buffering is required (Hollenbeck and Saxton, 2005; MacAskill and Kittler, 2010; Schwarz, 2013). The regulation of mitochondrial transport is particularly critical in polarized cells such as neurons. Compared to most other cell types, which are measured in micrometers, neurons extend their axons and dendrites for millimeters or centimeters, and in the case of human peripheral nerves up to a meter; therefore, disruption in the mitochondrial transport system often causes dysfunction in neurons, while other cell types appear unaffected (Baloh et al., 2007; Bossy-Wetzel et al., 2008; Chen and Chan, 2009; Johri and Beal, 2012; Reddy and Shirendeb, 2012). Lastly, mitochondrial quality control is necessary to maintain healthy mitochondria through a process called mitophagy, a form of autophagy by which defective mitochondria are selectively degraded (Ashrafi and Schwarz, 2013; Chen and Chan, 2009; Detmer and Chan, 2007b).

Mitochondrial dynamics are critical to mitochondrial functions and even mild defects in mitochondrial dynamics are associated with defective mitochondrial functions and disease. Mitochondrial dysfunction causes inability to respond to cellular energy needs, accumulation of

oxidative stress, cellular dysfunction, cell death, and disease. Mitochondrial dysfunction, including decreased oxidative capacity and increased oxidative damage, is also thought to contribute to aging. As organisms age, mitochondrial DNA mutations accumulate due to oxidative damage by reactive oxygen species (ROS), resulting in a decline in mitochondrial DNA volume, integrity, and function, as well as significantly increased ROS generation (Chistiakov et al., 2014). This concept is known as the "mitochondrial theory of aging," in which ROS accumulation causes oxidation of proteins, lipids, and nucleic acids, resulting in a decline in their cellular function (Harman, 1972). Interestingly, aging is also the largest risk factor for the development of neurodegenerative diseases, and patients and animal models of neurodegenerative diseases exhibit signs of mitochondrial dysfunction and oxidative stress implicating the mitochondrial theory of aging in the pathogenesis of neurodegenerative disorders (Cui et al., 2012). Accumulating evidence indicate that mitochondrial dysfunction is an early and causal event in the pathogenesis of neurodegenerative disorders such as HD, AD, ALS, and PD (Bossy-Wetzel et al., 2008; Johri and Beal, 2012; Knott et al., 2008; Lin and Beal, 2006). Mitochondrial dysfunction is also associated with an increasingly large number of inherited disorders in humans and is implicated in a myriad of common disorders such as cancer, autoimmune disorders, and metabolic syndromes (Lesnefsky et al., 2001; Pieczenik and Neustadt, 2007; Wallace, 1999). Both genetic mutations and exposure to environmental toxins can result in mitochondrial dysfunction and disease states. Therefore, a better understanding of the normal regulation of mitochondria and the consequences of mitochondrial dysfunction will provide novel insights into disease pathogenesis.

1.4.1. Structure of mitochondria

Mitochondria arose from a *Eubacterium* engulfed by a eukaryotic progenitor (Lane and Martin, 2010). Like their bacterial ancestor, the structure of mitochondria is composed of several distinct compartments and two separate membrane layers (Figure 1-4). The distinct mitochondria

subcompartments are as follows: the outer mitochondria membrane (OMM), the intermembrane space (IMS), the inner mitochondrial membrane (IMM), and the mitochondrial matrix. The IMS and the matrix are both aqueous compartments. The OMM separates the mitochondria from the cellular cytosol and is freely permeable to small molecules and ions less than 5 kDa, which move freely through transmembrane channels called porins. The IMM is only selectively permeable and contains the components of the respiratory chain and ATP synthase. The mitochondrial matrix contains all of the fuel oxidation pathways except glycolysis, which takes place in the cytosol, including the pyruvate dehydrogenase complex, and the enzymes for the citric acid cycle, the fatty acid β-oxidation pathway, and the amino acid oxidation pathways (Lehninger et al., 2005). The cristae membrane is imbedded in the IMM and is composed of IMM that folds into the matrix to increase the surface area for the electron transport chain (ETC). The region between the cristae folds is called the intercristae space and is where cytochrome c is located. Cytochrome c is essential to the ETC and for the signaling of apoptosis (Garrido et al., 2006). The ultrastructure of mitochondria and the proper targeting of mitochondrial proteins to the correct compartment is essential to mitochondrial function and signaling (Zick et al., 2009).

Like their bacterial ancestor, mitochondria have their own circular genome that is organized into discrete nucleoids in the mitochondrial matrix (Anderson et al., 1981; Pakendorf and Stoneking, 2005). In animals, mtDNA is almost exclusively inherited maternally, and paternal mtDNA is actively destroyed after fertilization (Al Rawi et al., 2011; Sato and Sato, 2011). During evolution the mitochondrial genome has been reduced though gene transfer to the nucleus and proteomic, genomic, and bioinformatic approaches have found that mammalian mitochondria contain over 1,500 proteins but the mitochondrial genome (mtDNA) only encodes about 1% of the mitochondrial proteins, or 13 polypeptides in humans (Gaston et al., 2009; Mootha et al., 2003; Pagliarini et al., 2008; Sickmann et al., 2003). The “mitochondria theory of aging” is based on the fact that mtDNA has a higher rate of mutation and less efficient repair machinery compared to nuclear DNA. MtDNA is exceptionally sensitive to oxidation owning its

close proximity to the source of ROS at the IMM and because mtDNA is not protected by histones (Richter et al., 1988). A mouse model with mutated mtDNA polymerase has an increased rate of mtDNA mutations and age prematurely, supporting a role for ROS in compromised mitochondrial functions in aging (Trifunovic et al., 2004). The 13 mtDNA encoded proteins are components of the ETC and are translated on mitochondrial ribosomes while other mitochondrial proteins, including other proteins required for the ETC, are encoded by nuclear DNA. Nuclear encoded mitochondrial proteins are transcribed on cytosolic ribosomes and imported into mitochondria. Targeting of nuclear encoded proteins can be through a mitochondrial targeting sequence (MTS), which is cleaved once these proteins enter the mitochondrial matrix (Omura, 1998). The MTS is a highly degenerate sequence typically composed of 15-40 amino acid residues and is rich in positively charged and hydroxylated amino acids, and is predicted to form α-helices or β-sheets which are important for their recognition by the translocation machineries in the OMM (Translocase of the outer membrane, TOM complex) and IMM (Translocase of the inner membrane, TIM complex) (Glover and Lindsay, 1992). Proteins can also be localized to mitochondria through interaction with mitochondrial lipids or mitochondrial proteins (Lindsay et al., 2011).

1.4.2. Functions of the mitochondria

Mitochondria are essential in carrying out the important cellular functions of ATP production, calcium buffering, and regulation of cell death through apoptosis (Gunter et al., 2004; Nunnari and Suomalainen, 2012; Parsons and Green, 2010). In eukaryotes, mitochondria use electron transport proteins, carriers, pumps, and enzymes to convert nutrients from food into ATP. The major pathway for ATP synthesis is oxidative phosphorylation (OXPHOS), which requires chemical intermediates from glycolysis and fatty acid oxidation metabolic pathways. Both of these pathways feed into the citric acid cycle, which in turn feed into the OXPHOS pathway. The electron transport chain (ETC) is composed of four protein machines, complexes I-IV, that are

located on the IMM (Lehninger et al., 2005). The ETC acts through sequential redox reactions that pump protons from the matrix to the IMS. Complexes I and II catalyze electron transfer to ubiquinone. Complex III carries electrons from reduced ubiquinone to cytochrome c. Complex IV transfers electrons from cytochrome c to O_2, completing the sequence (Lehninger et al., 2005). Complex I, also called NADH dehydrogenase, catalyzes electron transfer from NADH and a proton from the matrix to ubiquinone and couples the energy from that reaction to drive the transfer of protons from the matrix to the IMM. Therefore, Complex I is effectively a proton pump driven by the energy of electron transfer to move protons from the matrix to the IMS, making the matrix negatively charged and the IMS positively charged (Figure 1-4). Complexes III and IV also contribute to the generation of a proton gradient. Ubiquinol, the reduced form of ubiquinone diffuses in the IMM from Complex I to Complex III. Complex II, also called succinate dehydrogenase, is part of the citric acid cycle and catalyzes electron transfer from succinate to reduce ubiquinone. Complex I and II generate ROS, including hydrogen peroxide (H_2O_2) and superoxide radicals ($^{\cdot}O2^{-}$), which can damage cellular components such as proteins, lipids, and DNA (Chen et al., 2003b). Complex III, also called ubiquinone: cytochrome c oxidoreductase, couples the transfer of electrons from ubiquinol to cytochrome c, with the transport of protons from the matrix to the IMS. Cytochrome c then moves to complex IV. Complex IV, also called cytochrome oxidase, carries electrons from cytochrome c to molecular oxygen, reducing it to H_2O. The activity of Complex IV contributes to the electrochemical potential by pumping one proton outward into the IMS from the matrix and consuming four H^+ from the matrix in converting O_2 to H_2O. The proton gradient generated by complexes I, II, IV are used by ATP synthase or complex V, to drive phosphorylation of ADP to ATP (Lehninger et al., 2005). Defects in the ETC results in increased ROS production and decreased ATP production. Reduced ATP levels are linked to neurodegeneration and can result in cell death (Cho et al., 2010; Lin and Beal, 2006; Martin, 2001).

Calcium ion (Ca^{2+}) uptake into the mitochondria matrix is critically important to cellular function. Ca^{2+} is an important second messenger that affects numerous cell signaling pathways including synaptic transmission, muscle contractions, cell migration, cell growth, and cytokinesis (Gunter et al., 2004). The endoplasmic reticulum (ER) is the major intracellular Ca^{2+} store and the lumen of the ER contains a high concentration of free Ca^{2+} (100-500 μM) relative to the cytosol (~100 nM) (Berridge, 2002), and thus the regulated release of Ca^{2+} from the ER is essential for cellular signaling. Mitochondria, on the other hand, help regulate intracellular Ca^{2+} levels by taking up and releasing Ca^{2+} to control the spatiotemporal levels of Ca^{2+} in the cell and consequently mediate Ca^{2+} signaling and cellular response (Santo-Domingo and Demaurex, 2010). The Ca^{2+} buffering capacity of mitochondria has important consequences in localizing Ca^{2+} signaling to specific microdomains and depending on the cellular context can potentiate or inhibit Ca^{2+} signaling. For instance, in pancreatic acinar cells, mitochondria act as a barrier to prevent the propagation of cytosolic Ca^{2+} signal originating in the apical region of the cell, where secretion is regulated, from entering the basolateral part of the cell (Park et al., 2001; Tinel et al., 1999). In HeLa cells, mitochondria recycle Ca^{2+} to the ER to replenish ER Ca^{2+} stores (Arnaudeau et al., 2001) and can also transport Ca^{2+} directly from the plasma membrane to the ER avoiding Ca^{2+} entry into the cytosol (Jousset et al., 2007). Biochemically, a fraction of the ER can be isolated that is attached to mitochondria, referred to as the mitochondria-associated membrane (MAM), and stable ER-mitochondria contact sites are important biologically not only in coordinating Ca^{2+} transfer, but also in regulation of lipid synthesis, and control of mitochondrial fission (Rowland and Voeltz, 2012).

Ca^{2+} can freely cross the OMM, as the OMM is permeable to solutes that are smaller than 5 kDa due to the abundant expression of voltage-dependent anion channels (VDAC), a porin ion channel (Rizzuto et al., 2012). Uptake of Ca^{2+} into the mitochondrial matrix requires the mitochondrial Ca^{2+} uniporter (MCU) located on the IMM whereas Ca^{2+} can leave mitochondria through the mitochondrial permeability transport pore (MPTP) (Rizzuto et al., 2012) (Figure 1-4).

Ca^{2+} flux into mitochondria is dependent on mitochondrial membrane potential ($\Delta\Psi_m$) and the MCU catalyzes the passive transport of Ca^{2+} across the IMM using the negative $\Delta\Psi_m$ generated by the electron transport chain (Santo-Domingo and Demaurex, 2010). Ca^{2+} is also a key regulator of mitochondrial function and is required for OXPHOS. Ca^{2+} activates three matrix dehydrogenases: pyruvate dehydrogenase, α-ketoglutarate, and isocitrate-dehydrogenase and stimulation of these dehydrogenases by Ca^{2+} can increase ATP synthesis to match the energy needs of the cell (Jouaville et al., 1999). Mitochondrial dysfunction can affect multiple cellular processes that rely on Ca^{2+} signaling and dysregulaion of mitochondrial Ca^{2+} homeostasis is pathological. The overload of mitochondrial matrix Ca^{2+} levels can lead to enhanced production of ROS, cytochrome c release, and trigger apoptosis. In neurons, Ca^{2+} signaling is critical as Ca^{2+} levels are closely tied to synaptic transmission and synaptic plasticity (Billups and Forsythe, 2002; David and Barrett, 2003). The polarized structure of neurons and the large ATP requirement at distal neuronal processes highlights the necessity for mitochondrial Ca^{2+} sensing and mitochondrial distribution in neurons. Defects in Ca^{2+} levels are implicated in neurodegenerative disorders (de Brito and Scorrano, 2008; Panov et al., 2002; Rizzuto et al., 2012).

Apoptosis is the major type of regulated cell death pathway. Mitochondria can sense homeostatic fluctuations and various types of toxic insults including UV radiation, starvation, DNA damage, and chemotherapeutic agents to initiate the intrinsic apoptosis pathway (Fulda et al., 2010; Hardwick and Soane, 2013). In response to some lethal stimuli, mitochondria can undergo mitochondrial outer membrane permeabilization (MOMP), leading to release of cytochrome c from the mitochondrial intermembrane space into the cytoplasm (Garrido et al., 2006; Parsons and Green, 2010). Released cytochrome c then binds apoptotic protease-activating factor 1 (Apaf-1) inducing its conformational change and leading to the formation of the apoptosome, a caspase activating center (Tait and Green, 2010). The apoptosome activates the initiator caspase, caspase 9, which in turn activates other caspases to execute the cell death

cascade. MOMP is a highly regulated process controlled through the interaction of pro- and anti-apoptotic B cell lymphoma 2 (Bcl-2) family members (Hardwick and Soane, 2013). Activation of Bcl-2 protein BAX or BAK is essential for MOMP. Activation of BAX or BAK is controlled though interaction with other members of the Bcl-2 family such as tBID, and activated BAX or BAK interacts with voltage-dependent anion channel (VDAC) on mitochondria and causes the release of cytochrome c and other mitochondrial proteins from the mitochondrial intermembrane space into the cytosol. Cells that have been depleted of mitochondria through widespread induction of mitophagy cannot undergo apoptosis (Tait et al., 2013). Dysregulation of the signals regulating apoptosis can lead to cell death and disease. Abnormal apoptosis plays a role in the etiology of cancer, autoimmune disease, and neurodegenerative disorders. Chemotherapeutic agents act by inducing apoptosis in cancer cells. Conversely, abnormally increased activation of the apoptotic cascade is found in neurodegenerative diseases (Martin, 2001; Yuan and Yankner, 2000), suggesting that unwarranted cell death contributes to pathogenesis of neurodegenerative disorders.

1.4.3. Mitochondrial dynamics: fusion, fission, transport, and mitophagy

The term mitochondrial dynamics encompasses the processes of fusion, fission, transport and turnover to maintain a healthy mitochondrial network (Figure 1-3). These dynamic processes are essential to mammalian development. The classical depiction of mitochondria in textbooks is of a static bean-shaped structure but in living cells mitochondria are highly dynamic organelles that organize into a reticular network (Lewis and Lewis, 1914). Mitochondria can have different morphologies depending on cell type and adopt a range of morphologies that include small spheres, short rods, and long tubules. In fibroblasts, imaging studies using fluorescent probes against mitochondria indicate that mitochondrial diameter is about 0.5 μm, while their lengths are variable and can range from 1-10 μm or more (Detmer and Chan, 2007b; Okamoto and Shaw, 2005). Live cell imaging studies show that mitochondria are constantly in motion and are

transported with their long axis aligned along microtubule or actin tracks (Hollenbeck and Saxton, 2005). Mitochondrial transport is required to distribute mitochondrial functions throughout the cell. While mitochondria are being transported they can encounter each other and undergo fusion to merge their double membranes and mix their intramitochondrial contents. On the other hand, an individual mitochondrion can undergo fission to divide and yield two separate, shorter mitochondria (Ingerman et al., 2005; Knott et al., 2008). The frequencies of fusion and fission events are normally balanced in healthy cells at homeostasis. The perturbation of this balance through genetic mutations or pharmacological treatment leads to dramatic morphological changes (Knott and Bossy-Wetzel, 2008; Ruan et al., 2013; Santel and Fuller, 2001; Tondera et al., 2009; Yoon et al., 2001). Cells with a high fusion-to-fission ratio have very few individual mitochondria and these mitochondria are abnormally elongated and highly interconnected, a morphology referred to as "elongated mitochondria" or "hyperfused mitochondria." Cells with a high fission-to-fusion ratio have numerous small rod or sphere shaped mitochondria, a morphology referred to as "fragmented mitochondria." Maintenance of mitochondrial health is critically important for cytoprotection and cellular health; therefore, the maintenance of healthy mitochondria pool is essential. Mitophagy, the regulated turnover of mitochondria though autophagic degradation, is essential to dispose of damaged mitochondria (Ashrafi and Schwarz, 2013; Kim et al., 2007b). Defects in mitophagy results in accumulation of damaged mitochondria and elevated production of ROS, which contributes to disease.

1.4.4. Mitochondrial fusion

Mitochondrial fusion involves the merging of two individual mitochondria into one mitochondrion. The core machineries that mediate OMM and IMM fusion have been elucidated (Figure 1-3A). In mammals, there are two GTPases, Mitofusins Mfn 1 and Mfn 2, located on the OMM that are essential to OMM fusion. The Mitofusins are anchored to the OMM through the C-terminal segment and both the N-terminal GTPase domain and a C-terminal coiled-coil heptad

segment are in the cytoplasm (Fritz et al., 2001; Koshiba et al., 2004). The yeast homologue of the Mitofusins is *fuzzy onion* (Fzo1) and is required for mitochondrial fusion in yeast (Fritz et al., 2001). Mitofusins form homo-oligomeric and heter-oligomeric complexes on apposing mitochondrial outer membranes (Chen et al., 2003a; Meeusen et al., 2004). The heptad repeat region on Mitofusins form antiparallel coiled coils in trans between apposing mitochondria is thought to tether mitochondria during fusion (Koshiba et al., 2004), while GTPases activity drives membrane merging. Mice that lack Mfn1or Mfn2 do not survive past mid-gestation, due to placental defects (Chen et al., 2003a). Knockdown of Mfn1 or Mfn2 in cell culture results in mitochondrial fragmentation (Chen et al., 2003a; Eura et al., 2003).

Normally, OMM and IMM fusion occur in unison but can be experimentally uncoupled. In mammals, the GTPase optic atrophy type I (OPA1) is located on the IMM and is essential to IMM fusion. OPA1 and its yeast orthologue Mgm1 have an N-terminal MTS that is cleaved by a mitochondrial matrix protease once imported into mitochondria. In humans and mice, eight alternatively spliced isoforms are transcribed from the OPA1 gene that are imported into the mitochondrion, where the MTS is cleaved to produce long isoforms of OPA1 (l-OPA1) embedded in the IMM (Delettre et al., 2001; Song et al., 2007). L-OPA1 isoforms are cleaved at two different protease sites, S1 and S2, to produce short forms of OPA1 (s-OPA1) that are no longer anchored to the IMM. Both l-OPA1 and s-OPA1 isoforms are needed for fusion (DeVay et al., 2009; Herlan et al., 2003; Song et al., 2007), suggesting a high complexity in the regulation of IMM fusion. Mice that lack OPA1 do not survive past mid-gestation (Chen et al., 2003a; Davies et al., 2007). Knockdown of OPA1 in mammalian cell culture leads to mitochondrial fragmentation, reduced respiratory capacity, altered IMM and cristae morphology, and increased apoptosis (Arnoult et al., 2005; Frezza et al., 2006; Griparic et al., 2004; Lee et al., 2004a; Meeusen et al., 2006; Olichon et al., 2003). Over-expression of OPA1 in cell culture promotes mitochondrial hyperfusion (Cipolat et al., 2004). Mutations in OPA1 cause autosomal dominant optic atrophy (ADOA), the most common heritable form of optic neuropathy (Alexander et al.,

2000; Delettre et al., 2000). ADOA is characterized by degeneration of retinal ganglion cells of the optic nerve, leading to vision impairment and in many cases blindness. Over 100 pathogenic mutations in OPA1 have been reported, most of these mutations occur in the GTPase domain (Ferre et al., 2005).

Although the precise reason for mitochondrial fusion is not completely clear, mitochondrial fusion is thought to be cell protective and necessary to maintain mitochondrial functions. Fusion allows the exchange of contents between healthy and dysfunctional mitochondria (Chen and Chan, 2009; Chen et al., 2007; Chen et al., 2010a; Legros et al., 2002). This exchange or content mixing allows for the replacement or repair of damaged materials such as mtDNA. During starvation and cellular stress mitochondria elongate and hyperfuse to sustain ATP production, in a process termed stress-induced mitochondrial hyperfusion (SIMH) (Gomes et al., 2011; Rambold et al., 2011; Tondera et al., 2009). During starvation, hyperfused mitochondria are protected from degradation and from apoptosis. The genetic or pharmacological blockage of mitochondrial fusion causes stress-induced cell death, and therefore mitochondrial hyperfusion is cell protective.

Mutations in mitochondrial fusion proteins cause human disease. For example, mutations in Mfn2 cause Charcot-Marie Tooth type 2A (CMT2A) disease, a hereditary neuropathy, in which there is progressive motor and sensory impairment and degeneration of long peripheral axons. Over 40 mutations in Mfn2 are associated with CMT2A, and nearly all of the disease alleles cluster in or near the GTPase domain (Zuchner et al., 2004). Some of the CMT2A causing mutant forms of Mfn2 are unable to mediate mitochondrial fusion (Detmer and Chan, 2007a), while the exogenous expression of other disease causing mutant forms of Mfn2 lead to impairment of mitochondrial transport and distribution (Baloh et al., 2007; Misko et al., 2010). Although Mfn1 and Mfn2 are highly homologous proteins and both function in mitochondrial fusion, they also have divergent functions that may contribute to unique disease phenotypes. Mfn1 is more effective at driving mitochondrial fusion and is alone sufficient to mediate fusion

with OPA1 (Cipolat et al., 2004; Ishihara et al., 2004). Though knockout of either Mfn1 or Mfn2 in mice cause placental defects and embryonic lethality, conditional loss of Mfn1 outside of the placenta allows for normal development and survival into adulthood, while conditional loss of Mfn2 outside of the placenta causes early postnatal death and multisystem abnormalities (Chen et al., 2007). Recent studies have shown that Mfn2 has functions outside of mitochondrial fusion, including regulation of mitochondrial transport (Misko et al., 2010) and fusion of mitochondria with ER membranes (de Brito and Scorrano, 2008).

1.4.5. Mitochondrial fission

Mitochondrial fission involves the division of a single mitochondrion into two separate mitochondria (Figure 1-3B). Mitochondrial fission requires the recruitment of dynamin-related protein 1 (Drp1) in mammals and Dnm1 in yeast from the cytosol to mitochondria (Ingerman et al., 2005; Loson et al., 2013; Smirnova et al., 2001). During mitochondrial fission, Drp1 assembles into a ring around the circumference of the OMM and constricts to divide the mitochondrion (Figure 3-1B). The recruitment of Drp1 to OMM is dependent on four integral OMM proteins: Fission 1 (Fis1), Mitochondrial Fission Factor (Mff), and Mitochondrial Dynamics proteins of 49 and 51 kDa (MiD49 and MiD51) (Loson et al., 2013; Yoon et al., 2003). Fis1 is anchored to the outer mitochondrial membrane by a C-terminal hydrophobic tail (Yoon et al., 2003) and contains a tetratriopeptide repeat (TPR) motif domain facing the cytosol (Dohm et al., 2004; Suzuki et al., 2003). Human Fis1 is not localized specifically to the mitochondrial scission sites but circumscribes the OMM (Suzuki et al., 2003; Yoon et al., 2003). Mff is critical for recruitment of Drp1 and also regulates mitochondrial fission (Gandre-Babbe and van der Bliek, 2008; Loson et al., 2013; Otera et al., 2010). MiD49 and MiD51 also regulate Drp1 recruitment but may only induce fission under certain cellular contexts, such as during mitochondrial membrane depolarization (Loson et al., 2014; Loson et al., 2013). Additionally, the post-translational modification of Drp1 is required for recruitment to mitochondria and for fission

activity. The ubiquitination of Drp1 by the mitochondrial E3 ubiquitin ligase March5 is required for fission (Karbowski et al., 2007; Nakamura et al., 2006). Drp1 activity is also controlled by phosphorylation at Ser637 by PKA to inhibit fission (Chang and Blackstone, 2007a; Chang and Blackstone, 2007b) and dephosphorylation by calcineurin phosphatase promotes recruitment of Drp1 to mitochondria to promote fission (Cereghetti et al., 2008).

Mitochondrial fission is required during apoptosis, development, and cell division (Chang et al., 2010; Frank et al., 2001; Ishihara et al., 2009). Excessive mitochondrial fission is linked to mitochondrial dysfunction and cellular pathology (Chen et al., 2005; Knott et al., 2008). In humans, a single point mutation has been reported in Drp1 (A395D), which resulted in death at 37 days after birth. The patient displayed a wide range of abnormalities including abnormal brain development, increased lactic acid, and optic atrophy. Patient-derived fibroblasts showed elongated mitochondria and peroxisomes (Waterham et al., 2007). Expression of this mutant Drp1 in cell culture results in defects in mitochondrial and peroxisome fission (Chang et al., 2010), implicating disruption in mitochondrial fission in neonatal lethality. Furthermore, mice lacking Drp1 die during embryonic development, have defects in synapse formation, and developmental defects in the heart, liver, and nervous system (Ishihara et al., 2009). In cell culture, inhibition of Drp1 GTPase activity with a dominant negative protein defective in GTP binding ($Drp1^{K38A}$) results in mitochondrial hyperfusion and abnormal mitochondrial elongation (Frank et al., 2001; Smirnova et al., 2001). The knockdown of Fis1 by RNAi in cell culture also results in mitochondrial hyperfusion and elongation (Stojanovski et al., 2004).

1.4.6. Mitophagy

Mitophagy is the specific elimination of mitochondria by autophagy to regulate the number of mitochondria, to maintain quality control, and for steady-state turnover of mitochondria (Elmore et al., 2001; Kim et al., 2007b; Tal et al., 2007; Youle and Narendra, 2011). Generalized autophagy (macroautophagy) is a process for the bulk catabolism of cellular

components, including proteins and organelles, by encapsulation in a double membrane vesicle called the autophagosome. The autophagosome fuses with lysosomes to allow degradation of the internal cargo and the inner bilayer of the double autophagosome membrane. Autophagy is important for the recycling of intracellular components and for cell survival during nutrient deprivation (Glick et al., 2010). Autophagy is regulated by autophagy-related (Atg) proteins, which over 30 have been identified (Klionsky et al., 2011). There are two ubiquitin-like systems that are key to autophagy and are required for autophagosome formation, the Atg5-Atg12 conjugation step and the Atg8/LC3 step (Glick et al., 2010). In mammalian cells, mitochondrial fission occurs upstream of mitophagy to segregate damaged mitochondria from healthy mitochondria as well as to generate mitochondria of appropriate size for autophagosome engulfment (Twig et al., 2008).

Excessive mitochondrial damage is linked to PD (Schapira, 2008). PD is one of the most common neurodegenerative disorders and is caused by the loss of dopaminergic neurons in the substantia nigra pars compacta, a region in the midbrain that is important for motor control (Riederer and Wuketich, 1976; Surmeier et al., 2010). In mammals, mitophagy of damaged mitochondria is regulated by parkin (PARK2), an E3 ubiquitin ligase, and the ser/thr kinase PTEN-induced putative kinase protein 1 (PINK1, PARK6) (Figure 1-3 C). Mutations in these proteins are linked to early onset autosomal recessive PD. Parkin is normally located in the cytosol but translocate specifically to damaged mitochondria. The recruitment of parkin to mitochondria is dependent on PINK1 (Kim et al., 2008; Matsuda et al., 2010; Sha et al., 2010), a protein that is rapidly and constitutively degraded in healthy mitochondria but stabilized on the OMM in response to loss of mitochondrial membrane potential in damaged mitochondria (Jin et al., 2010; Matsuda et al., 2010; Narendra et al., 2010). The induction of mitophagy is dependent on parkin E3 ligase activity and once parkin is recruited to mitochondria several OMM proteins are ubiquitinated by parkin, including Mfn1, Mfn2, Miro1, Miro2, and VDAC1 to trigger their degradation (Gegg et al., 2010; Geisler et al., 2010a; Tanaka et al., 2010). The degradation of

Mitofusins is thought to prevent fusion of damaged mitochondria with healthy mitochondria and the degradation of Miros is though to prevent the transport of damaged mitochondria once mitophagy has been initiated. PINK1 has also been shown to regulate mitochondrial motility by phosphorylation of Miro, a component of the mitochondria trafficking machinery. The interaction of PINK1 with Miro is dependent on loss of mitochondrial membrane potential, resulting in Miro phosphorylation at Ser156 by PINK1 to promote Miro degradation (Wang et al., 2011). This finding suggests that inhibition of mitochondrial motility may be required for efficient mitophagy. Parkin and PINK1 act in the same pathway to mediate mitochondria quality control to selectively remove damaged mitochondria and it is suggested that failure to clear normally occurring damaged mitochondria by mitophagy can cause PD. Furthermore, many of the pathogenic mutations of PINK1 and parkin fail to induce mitophagy (Geisler et al., 2010b; Matsuda et al., 2010), supporting the notion that failure to eliminate damaged mitochondria by mitophagy contributes to PD pathogenesis. Parkin has also been found to be a tumor suppressor (Cesari et al., 2003; Poulogiannis et al., 2010), suggesting that mitophagy regulation may also be important in cancer etiology.

1.4.7. Mitochondrial transport and regulation

The transport of mitochondria is important to distribute mitochondrial functions in a regulated and non-uniform fashion. The regulation of mitochondrial transport is particularly critical in neurons due to their asymmetrical shape and long axonal and dendritic processes. Correct mitochondrial dynamics seem to be particularly important to neuronal function and defects in mitochondrial transport are implicated in the pathogenesis of several major neurodegenerative disorders (Baloh et al., 2007; Chen and Chan, 2009; Detmer and Chan, 2007b; Hirokawa et al., 2010; Hollenbeck and Saxton, 2005; Ikenaka et al., 2012; Karle et al., 2012; Liu et al., 2012; Reddy and Shirendeb, 2012). After biogenesis in the cell body, proteins and organelles must be transported into the axon and synaptic terminals by anterograde transport

(Grafstein and Forman, 1980; Hollenbeck and Saxton, 2005). After serving critical functions in the axon, aged proteins and organelles can accumulate oxidative damage and must be returned to the cell body for degradation by retrograde transport. This idea that new healthy mitochondria are transported from the cell body to distal regions while damaged, aged mitochondria are transported to the cell body for degradation is supported by the finding that mitochondria with high membrane potential move in the anterograde direction, while mitochondria with low membrane potential move in the retrograde direction (Cai et al., 2012; Miller and Sheetz, 2004). Furthermore, damaged mitochondria must be retrogradely transported to the cell body for mitophagy, as mature lysosomes are predominately localized in the cell body (Cai et al., 2012). Live cell imaging studies indicate that during long distance transport of mitochondria in axons, mitochondrial movement is dynamic with mitochondria moving bi-directionally, with brief pauses, and frequent changes in direction but strongly biased towards one direction (Misgeld et al., 2007; Morris and Hollenbeck, 1995; Pilling et al., 2006). The mean velocity of neuronal mitochondria is highly variable ranging from 0.32 to 0.91 μm/sec (MacAskill and Kittler, 2010).

Long distance and rapid transport of mitochondria occurs along the microtubule network, while actin serves as tracks for short-range transport in areas beyond the reach of microtubules (Hollenbeck and Saxton, 2005; Morris and Hollenbeck, 1995). In axons, microtubules are organized uniformly with their plus ends oriented towards the synaptic terminals, whereas axons contain short actin filaments that are of mixed polarity (Bearer and Reese, 1999; Fath and Lasek, 1988; Morris and Hollenbeck, 1995). Myosin motors direct the movement of mitochondria on actin filaments towards both the plus end and the minus end (Hollenbeck and Saxton, 2005), while kinesin motors move mitochondria towards microtubule plus ends in a process called anterograde transport and dynein motors move mitochondrial towards microtubule minus ends in a process called retrograde transport (Hollenbeck and Saxton, 2005; Pilling et al., 2006; Schwarz, 2013). Dendritic microtubules exhibit mixed polarity and so kinesin and dynein motors can drive cargo transport in dendrites towards and away from the cell body depending on the microtubule

polarity (Kapitein et al., 2010a). Each kinesin motor contains two heavy chains that have a head domain, which generates motion though ATP hydrolysis (Carter et al., 2011; Rayment et al., 1993; Rice et al., 1999). The head domains are connected by a stalk sequence, which allow the motors to walk on microtubules with alternative cycles of head domain binding to microtubules, so that one head domain is always attached to the microtubule (Vale and Milligan, 2000). Members of the kinesin-1 family, collectively termed KIF5, are the main motors driving anterograde mitochondrial transport in neurons (Hurd and Saxton, 1996; Pilling et al., 2006; Tanaka et al., 1998). In mammals there are three KIF5 isoforms: KIF5A, KIF5B, and KIF5C. KIF5B is ubiquitously expressed, whereas KIF5A, and KIF5C expression is restricted to neurons (Hirokawa et al., 2010). The targeted deletion of KIF5A or KIF5B in mice disrupts mitochondrial transport and results in perinuclear accumulation of mitochondria (Karle et al., 2012; Tanaka et al., 1998). Multiple lines of evidence suggest that different classes of motor proteins are simultaneously bound to mitochondria and function cooperatively. For example, kinesin-1 mutations inhibit both anterograde and retrograde transport of mitochondria (Pilling et al., 2006) and mitochondria can rapidly reverse their direction of transport. These findings suggest that mitochondria may simultaneously engage both kinesin and dynein motors, although how motor switching is regulated is unknown (Hirokawa et al., 1990; Ligon et al., 2004; Sheng and Cai, 2012; Welte, 2004). The major motor that drives retrograde mitochondrial transport in axons is cytoplasmic dynein (Pilling et al., 2006; Schnapp and Reese, 1989; Waterman-Storer et al., 1997). It contains multiple subunits, including two catalytic heavy chains (DHC), several intermediate chains (DIC), several light intermediate chains (DLIC), and several light chains (DLC). Dynein motor activity also requires interaction with dynactin complex though the subunit $p150^{Glued}$ (Kardon and Vale, 2009; McKenney et al., 2014; Moughamian et al., 2013; Pilling et al., 2006).

The key conserved and major mitochondrial anterograde transport machinery is the Miro/Trak1 (Milton) complex (Figure 1-3D). Miro 1 and Miro 2 were originally identified in mammals as atypical Rho-like small GTPases (Fransson et al., 2003). The Miro proteins contain

two GTPase domains, two EF-hand Ca^{2+}-binding domains, and a C-terminal transmembrane domain, which is anchored to the OMM (Fransson et al., 2003; Fransson et al., 2006; Frederick et al., 2004). Trak1 and its homolog GRIF-1 can both interact with Miro proteins and both Trak1 and GRIF-1 also directly bind to kinesin motors, acting as an adaptor linking mitochondria to microtubules to facilitate their transport (Brickley et al., 2005; Smith et al., 2006). Recently, dynein was also found to interact with the Trak1 and with GRIF1 (Russo et al., 2009; van Spronsen et al., 2013) and dynein was found localized to mitochondria moving in both the anterodrade and retrograde directions (van Spronsen et al., 2013). An interesting possibility is that the Miro/Trak1 complex can coordinately regulate dynein and kinesin transport. Trak1 and GRIF-1 have been shown to have different roles in regulating mitochondrial transport in axons and dendrites through different affinities to kinesin and dynein motors. Trak1 has been shown to bind both KIF5 and dynein and regulate mitochondrial transport in axons, while GRIF-1 predominately binds dynein and mediates mitochondrial transport in dendrites (van Spronsen et al., 2013). In resting, non-polarized cells Miro facilitates mitochondria transport by linking mitochondria to kinesin motors though interaction with Trak1/GRIF-1 (Brickley and Stephenson, 2011; MacAskill et al., 2009a). However, during conditions of elevated Ca^{2+} levels, Miro's EF hands bind Ca^{2+} and halt mitochondrial transport, thus allowing mitochondrial movement to be regulated by calcium levels to position mitochondria near Ca^{2+} sources to enhance Ca^{2+} buffering and ATP production where needed (Wang and Schwarz, 2009). The knockdown of Miro results in decreased sensitivity to Ca^{2+} inhibition of mitochondrial motility while over-expression of Miro results in increased sensitivity to Ca^{2+} inhibition of mitochondrial motility, suggesting a direct relationship between Miro levels and sensitivity to Ca^{2+} levels on mitochondrial transport (Saotome et al., 2008). The molecular mechanism for uncoupling mitochondria from transport machinery by Miro mediated Ca^{2+} sensing is not known. A proposed model suggests that under low Ca^{2+} levels Miro interacts with Trak1 and is therefore linked to kinesin and microtubules. However, an increase in Ca^{2+} is proposed to cause the kinesin motor domain to dissociate from

microtubules and bind Miro instead, cutting off mitochondria from microtubule tracks. Trak1 has also been shown to be O-GlcNAcylated by the enzyme O-GlcNAc transferase (OGT) in response to elevated extracellular glucose to immobilize mitochondria, suggesting that Trak1 activity can be modulated by glucose levels (Pekkurnaz et al., 2014). These finding suggests that Miro/Trak1 complex can coordinate regulation of mitochondrial trafficking in response to various intracellular signaling events.

Depletion of either Trak1 or Miro proteins greatly reduces mitochondrial motility but does not completely abolish mitochondrial motility (Brickley and Stephenson, 2011; Guo et al., 2005; Macaskill et al., 2009b; Saotome et al., 2008; van Spronsen et al., 2013), suggesting that there are other mitochondrial transport machineries. Syntabulin, a mitochondrial OMM protein that contains a mitochondrial targeted carboxyl-terminal transmembrane domain also acts as a KIF5 adaptor through direct binding with KIF5 (Cai et al., 2005; Su et al., 2004). In cultured neurons, knockdown of syntabulin results in reduced transport of mitochondria to neuronal processes and clustering in the cell body (Cai et al., 2005). Silencing fasciculation and elongation protein 1 (FEZ-1) has also been shown to act as an adaptor connecting mitochondria to KIF5 in PC12 cells (Fujita et al., 2007). In non-neuronal cells RNA-binding protein 2 (RanBP2) has been shown to act as a mitochondrial adaptor, and can interact with KIF5B and KIF5C (Cho et al., 2007; Patil et al., 2013).

In mature neurons, only about 20-30% of axonal mitochondrial are in motion, while about 15% of mitochondria are docked at synapses (Chen and Sheng, 2013; Kang et al., 2008). The anchored mitochondria at presynaptic terminals have been shown to provide a continuous and stable supply of ATP (Sun et al., 2013). Syntaphilin binds microtubules and associates with stationary but not motile mitochondria, and is suggested to act as a docking protein to anchor mitochondria at a particular location (Chen and Sheng, 2013; Chen et al., 2009; Kang et al., 2008). Syntaphilin is an OMM protein with a carboxyl-terminal mitochondrial targeting domain and is only localized to axonal mitochondria. In mice, deletion of syntaphilin results in strongly elevated

levels of motile mitochondria, while over-expression of syntaphilin abolished mitochondrial transport (Kang et al., 2008).

A growing number of observations suggest that the regulation of mitochondrial transport appears to be critical to mitochondrial fusion-fission dynamics. These include reports that over-expression of Miro or Trak1/Milton increases rates of mitochondrial fusion to generate hyperfused mitochondria (Fransson et al., 2006; Koutsopoulos et al., 2010; MacAskill et al., 2009a; Russo et al., 2009; Saotome et al., 2008); the knockdown of mitochondrial fission protein Drp1 reduces the transport rate of mitochondria (Verstreken et al., 2005). The mitochondrial fusion protein Mfn2 has been shown to be directly involved in and required for axonal mitochondrial transport in both the anterograde and retrograde directions via formation of a complex with Miro/Trak1 (Baloh et al., 2007; Misko et al., 2010). The disruption of Mfn2 results in selectively diminished mitochondrial transport, while the transport of other organelles is not affected. Both Mfn1 and Mfn2 interact with mammalian Miro proteins (Miro1 and Miro2) and with Trak1 (Milton/GRIF-1) (Misko et al., 2010), suggesting that Mitofusin proteins, particularly Mfn2, are also regulatory agents in mitochondrial trafficking. These data highlight the notion that mitochondrial transport and fusion may be coordinately regulated processes that are intimately linked and it is likely that the molecular adaptors Trak1 (and related protein GRIF-1) and Miro serve dual roles to coordinately regulate transport and fusion. In neurons, mitochondria in the cell body exist in a reticular network but in axons are isolated individual bean shaped organelles, suggesting that small, individual mitochondria are required for transport and that precise control of fusion/fission is needed to maintain appropriate mitochondrial size for transport. In fact mitochondria that are abnormally long due to experimental perturbation do not move (Amiri and Hollenbeck, 2008). Interestingly, Miro was found to interact with PINK1 and parkin and is ubiquitinated and degraded upon mitochondrial damage (Liu et al., 2012; Wang et al., 2011; Weihofen et al., 2009), suggesting that during mitophagy Miro is degraded to terminate Miro/Trak1-mediated transport pathways and that termination of mitochondrial transport is

important for mitophagy. Taken together, mitochondrial dynamics are inter-related processes that reciprocally influence one another and together fusion, fission, transport, and mitophagy are part of a complex, interacting pathway that controls mitochondrial function and cellular health.

1.4.8. Consequences of mitochondrial dysfunction

Mitochondrial dysfunction results in the inability to respond to the cell's energy needs, aberrant Ca^{2+} signaling, and improper apoptotic cell death. In addition to impaired mitochondrial functions, mitochondrial dysfunction can also result in the production of toxic ROS, a major cause of cellular oxidative stress, which can damage cellular proteins, lipids, and DNA, disrupting their function (Cui et al., 2012; Kirkinezos and Moraes, 2001). Neurons are particularly dependent on proper mitochondrial dynamics and are particularly sensitive to mitochondrial dysfunction, supported by the role of mitochondrial dysfunction in the pathogenesis of neurodegenerative diseases (Alexander et al., 2000; Johri and Beal, 2012; Kijima et al., 2005; Knott et al., 2008; Reddy and Shirendeb, 2012). Unlike other cell types, neurons are post mitotic cells that survive for the lifetime of the organism and are non-regenerative. The brain consumes twenty percent of the body's resting energy even though the brain makes up only 2% of the body's weight. This high amount of energy consumption is due to action potential signaling and synaptic transmission (Harris et al., 2012), which require mitochondria to constantly produce energy for these functions. Neurons strongly rely on OXPHOS due to their limited capacity for glycolysis (Bolanos et al., 2010; Herrero-Mendez et al., 2009). Furthermore, neurons are highly polarized and have elongated processes that are distant from the cell body, requiring the long distance trafficking of mitochondria. Defective mitochondrial trafficking is increasingly implicated in neurodegenerative diseases (Baloh et al., 2007; Hirokawa et al., 2010; Ikenaka et al., 2012; Karle et al., 2012; Liu et al., 2012). Morphological alternations of mitochondria have been observed in multiple pathologies, suggesting a close relationship between mitochondrial structure and cellular health. In normal cells mitochondria display a dynamic tubular structure that is

constantly undergoing fusion, fission, transport, and turnover; however, pathology arises when the dynamic nature of mitochondria are disrupted.

In humans, mutations in the OMM fusion protein Mfn2 lead to the neurodegenerative disease CMT2A (Kijima et al., 2005), while mutations in IMM fusion protein, OPA1, lead to the neurodegenerative disease dominant optic atrophy (Alexander et al., 2000; Carelli et al., 2004; Davies et al., 2007). Mutations in PINK1 and parkin, two proteins involved in the regulation of mitophagy are implicated in autosomal recessive early onset PD (Geisler et al., 2010b). Both sporadic and familial forms of PD involve mitochondrial dysfunction in disease pathogenesis (Schapira, 2008; Surmeier et al., 2010; Trimmer et al., 2000; Winklhofer and Haass, 2010). The PD associated genes also include DJ-1 and α-synuclein, which have been implicated in mitochondrial function. Mutations in α-synuclein are associated with autosomal dominant familial PD. A population of α-synuclein is localized to mitochondria and ER-mitochondria contact sites (Auluck et al., 2010; Devi et al., 2008; Guardia-Laguarta et al., 2014; Parihar et al., 2008) and over-expression of wild-type α-synuclein results in mitochondrial fragmentation (Nakamura et al., 2011). In PD, mutant α-synuclein forms aggregates that are the major component of Lewy bodies. Mutations in DJ-1 are linked to autosomal recessive early onset PD (Bonifati et al., 2003) and DJ-1 is partially localized to mitochondria (Junn et al., 2009), and functions to protect against oxidative-stress induced cell death. Knockdown of DJ-1 results in mitochondrial fragmentation (Irrcher et al., 2010) and increased ROS levels (Giaime et al., 2012). In a small number of cases, inherited mtDNA mutations have been linked to parkinsonism (Luoma et al., 2004; Simon et al., 1999). Mitochondria were first implicated in PD pathogenesis due to the finding that MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) causes parkinsonism in drug users, due to the effect of MPTP on inhibition of Complex I of the mitochondria electron transport chain (ETC), and this model can be reproduced in laboratory animals (Fornai et al., 2005).

There is a wealth of literature supporting a role for mitochondrial dysfunction and oxidative damage in the pathogenesis of AD (Baloyannis, 2006; Chen and Chan, 2009; Johri and Beal, 2012; Pieczenik and Neustadt, 2007; Trimmer et al., 2000). There is evidence that mtDNA may be involved in AD pathogenesis; when patient mtDNA is transplanted into mtDNA deficient cell lines, these cells develop respiratory enzyme deficiencies seen in the brains and other tissues of Alzheimer's patients (Swerdlow et al., 1997). Moreover, it has been shown that many of the disease-related proteins interact with mitochondria. Amyloid precursor protein (APP) has a dual ER/mitochondria targeting sequence and overexpression of APP blocks mitochondrial protein import pathways causing impaired ATP production (Anandatheerthavarada et al., 2003). Aβ binds to a mitochondrial-matrix protein called Aβ-binding alcohol dehydrogenase (ABAD) and this interaction inhibits cytochrome oxidase activity and causes increased ROS generation (Lustbader et al., 2004; Manczak et al., 2006). Tissue samples from the spinal cord and muscles of patients with ALS show abnormalities in mitochondria structure and localization. It has also been reported that mutant Cu/Zn-super-oxide dismutase (SOD1), which is linked to 20% of familial cases of ALS, abnormally localizes to mitochondria (Liu et al., 2004), and that overexpression of the ALS linked G93A SOD1 mutation in a transgenic mouse model caused impaired mitochondrial energy metabolism in the brain and spinal cord (Mattiazzi et al., 2002). Multiple lines of evidence have shown involvement of mitochondrial dysfunction in HD, including decreased activities of Complex II of the ETC (Gu et al., 1996), and impaired ATP production (Milakovic and Johnson, 2005). There is also evidence that huntingtin protein directly interacts with mitochondria on the OMM (Choo et al., 2004; Panov et al., 2002).

Mitochondrial dysfunction and oxidative stress occur in the early stages of all major neurodegenerative diseases, and may have a causal role in disease pathogenesis. Many of the disease-linked proteins have been shown to interact with mitochondria or mitochondrial proteins, suggesting that these mutant proteins may directly impair mitochondria. One of the fundamental questions in understanding theses diseases is how certain tissues or particular populations of

neurons are selectively vulnerable to inherited mutations that regulate mitochondria dynamics in all tissues. One likely possibility is that neurons are more sensitive to perturbations in mitochondrial functions than other cell types.

Cancer cells are characterized by higher rates of cell proliferation and defects in apoptosis and other cell death signaling pathways (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011). Cancer cells adopt aberrant metabolic advantages that are collectively referred to as the "Warburg effect," in which they boost dependence on aerobic glycolysis for energy production and suppress OXPHOS to evade apoptosis. Normal cells utilize mitochondrial OXPHOS to generate 30 ATPs per glucose molecule while cancer cells heavily rely on glycolysis to generate 2 ATPs per glucose in the cytoplasm. Cancer cells have been shown to upregulate glucose transporters to increase glucose uptake into the cell to maintain hyperactive glycolytic pathways (Bhat et al., 2015; Jang et al., 2013). Hyperactive glycolysis is inhibitory to OXPHOS, which in turn disrupts mitochondria mediated cell death pathways thereby allowing cancer cells to escape cell death and increase cancer progression. Mitochondria in cancer cells are found to be hyperpolarized and have reduced ROS production and upregulation of anti-apoptotic Bcl-2 proteins (Bonnet et al., 2007; Chen, 1988; Sun et al., 2008). Metabolic inhibitors such as 2-deoxy-D-glucose (2DG), dichloroacetate (DCA), hexokinase inhibitors, and lactate dehydrogenase inhibitors have been used successfully in cancer therapies to block aerobic glycolysis and restore OXPHOS to induce apoptosis of cancer cells (Bonnet et al., 2007; Kankotia and Stacpoole, 2014; Zhao et al., 2013b).

1.5. Trak1 mutations are linked to disease states

Mutations in Trak1 protein have been linked to several diseases, including hypertonia in mice (Gilbert et al., 2006) and childhood absence epilepsy (Chioza et al., 2009) and gastric and colorectal cancers in humans (An et al., 2011; Zhang et al., 2009). The molecular and cellular pathogenic mechanisms of Trak1 mutations in disease etiology are unknown.

1.5.1. Hypertonia

Hypertonia is a pathophysiological movement disorder, characterized by spasticity. The primary diagnostic characteristic is resistance of muscles to stretch and increased tightness of muscle tone. In clinical terms, hypertonia is assessed as "resistance to passive stretch while the patient maintains a relaxed state of muscle activity" (Sanger et al., 2003). The regulation of muscle tone is needed to maintain normal posture and to support movement and balance. Spasticity occurs when there is increased stretch reflex, resulting in excessive and inappropriate muscle activation (Bar-On et al., 2015). The causes of hypertonia are wide-ranging and can results from traumatic brain and spinal cord injuries to molecular defects that lead to motor pathway abnormalities that either increase motor neuron excitability or reduce inhibitory mechanisms (Gilbert et al., 2006).

Hypertonia is associated with a number of human neurological disorders, including cerebral palsy, PD, dystonia, stroke, spastic paraplegia, epilepsy, and stiff person syndrome (Webber et al., 2008). The abnormal muscle activation, spasticity, and limb tremor associated with hypertonia contribute to disability and poor quality of life, with current treatments being limited (Richardson et al., 2000). Hypertonia is most commonly treated by manual stretching of affected muscles and antispasticity medications, such as botulinum toxins and drugs that act on the γ-aminobutyric acid (GABA)ergic system including baclofen, gabapentin, and benzodiazepines (Nair and Marsden, 2014; Richardson et al., 2000; Turner-Stokes and Ward, 2002). Botulinum toxins are injected into the affected muscles and produce a partial denervation and paralysis by blocking presynaptic release of the neurotransmitter acetylcholine at the neuromuscular junction (Richardson et al., 2000). Chemical denervation is reversible so the effects of botulinum toxins are temporary, and repeated treatment is required. Baclofen acts as a GABA agonist on $GABA_B$ receptors causing neuronal inhibition (Meythaler et al., 2001). Gabapentin is an anticonvulsant drug with a chemical structure similar to GABA but does not bind to GABA receptors and has no identified GABA receptor activity (Priebe et al., 1997). The

mechanism is suggested to be through the inhibition of glutamatergic transmission specifically through the inhibition of presynaptic glutamate release (Rabchevsky et al., 2011). Gabapentin is approved for the treatment of epilepsy and is widely used for the treatment of neuropathic pain. Benzodiazepines potentiate postsynaptic $GABA_A$ receptors to enhance GABAergic transmission (Campo-Soria et al., 2006). Treatment response is highly variable and although these drugs have been used for several decades, their efficacies are poor with limited evidence of their effectiveness.

Trak1 mutation is linked to severe hypertonia in mice. A spontaneous frame shift mutation in Trak1 resulting in deletion of the C-terminal portion of the protein was found to cause a recessively transmitted form of hypertonia in mice (Gilbert et al., 2006). At two weeks of age, onset of motor defects are clearly seen and include postural abnormalities, stiffness, jerky movements, limb tremor, and measurably elevated baseline firing of lower motor neurons by electromyography; these neurological symptoms are consistent with hypertonia in humans. Heterozygous animals appear normal and are indistinguishable from wild-type mice. Mouse Trak1 protein shares 92% overall amino acid identity with human Trak1 protein, with the main difference being an insertion of 12 amino acid residues (TVTSAIGGLQLN) after reside 896 in the human Trak1 sequence (Webber et al., 2008). Mice with homozygous hypertonia (hyrt) mutation in Trak1 have relatively normal lifespan and histology of the CNS did not reveal structural abnormalities, although inclusion bodies were found in the neuronal processes of the brain stem and spinal cord (Gilbert et al., 2006). EM analyses of spinal cord sections found that the inclusion bodies were composed of aggregates of vesicular structures, many of which had two-membrane layers. Accumulation of inclusion bodies and protein aggregates are associated with neurodegenerative disease. No ultrastructural abnormalities were found in the peripheral nerves, neuromuscular junctions, or muscles. No defects were observed in the number or morphology of motor neurons in hyrt mice, suggesting that motor neuron degeneration did not occur. However, hyrt mice did exhibit highly reduced expression of $GABA_A$ receptor α1 subunits,

with as much as 80% reduction for the spinal cord and brainstem and a 50-70% reduction in other CNS regions compared to wild-type mice. Protein levels did not seem to be affected for a variety of other neuronal receptors including, glycine, AMPA, kainate, or NMDA receptors.

In these mice, drugs such as Diazepam and Baclofen, which enhance GABAergic transmission, effectively ameliorated some hypertonia symptoms (Gilbert et al., 2006). It is yet unknown how the hyrt mutation disrupts $GABA_A$ receptor expression at the cell surface. Co-immunoprecipitation studies revealed that both Trak1 wild-type and Trak1 hyrt could precipitate $GABA_A$ receptor α1 subunit suggesting that the C-terminal truncation did not affect Trak1 interaction with $GABA_A$ receptor. However, Trak1 normally regulates the endosomal sorting of certain endocytosed cargo receptors (Webber et al., 2008). Given that the Trak1 hyrt mutation causes hypertonia when recessively transmitted suggests a loss-of-function mechanism. One possibility would be that Trak1 hyrt causes dysregulation of endosome-to-lysosome trafficking; however, our lab (Webber et al., 2008) failed to find any differences between Trak1 hyrt and Trak1 wild type in their localization or trafficking function. The colocalization of Trak1 hyrt with Hrs on early endosomes was indistinguishable from that of Trak1 WT. EGF receptor endocytic trafficking assays using the Trak1 hyrt mutation also failed to find any differences from that of Trak1 WT. The truncation responsible for Trak1 hyrt mutation is outside of the kinesin and Hrs binding domains, however, the mutation might disrupt binding of OGT enzyme. OGT enzyme binds to Trak1 at resides 639-859 (Iyer and Hart, 2003; Pekkurnaz et al., 2014) and the Trak1 hyrt deletion results in Trak1 truncation at amino acid 824; therefore, Trak1 hyrt may affect the post-translation modification of Trak1 at the C-terminus to affect its function. Additionally, it is also unknown how the hyrt mutation might affect mitochondrial trafficking or interaction with mitochondrial transport machinery. Trak1 is highly conserved in humans therefore, understanding the role of Trak1 may potentially play an important role in unraveling the molecular etiology of hypertonia and identifying more promising therapeutic targets for treatment of hypertonia symptoms.

1.5.2. Childhood absence epilepsy

Childhood absence epilepsy (CAE) is a type of idiopathic generalized epilepsy with multifactorial genetic etiology. CAE is a type of non-convulsive epilepsy characterized by frequent absence seizures that last for 4-20 seconds, and are marked by transitory loss of awareness or abrupt impairment of consciousness that is accompanied by a generalized 2.5-4 Hz spike and slow-wave discharge (SWD) on electroencephalogram (EEG) (Avoli et al., 2001; Crunelli and Leresche, 2002). Children undergoing a CAE event will typically "sit or stand with limbs relaxed staring vacantly, the eyeballs may roll upwards, the lids may flicker, but there are no convulsive movements"; the child losses awareness, becomes unresponsive, and arrests behavior during the CAE event and after the CAE event the child is immediately well and continues with the activity he was doing before the attack (Buchhalter, 2011). CAE typically start between 3 to 8 years of age, peaking at 6-7 years of age, and involves frequent absence seizures, sometimes up to ~200 per day (Crunelli and Leresche, 2002). In 70% of patients spontaneous remission occurs around adolescence (Panayiotopoulos, 2001).

The annual incidence of CAE has been reported in the range of 2-8 cases per 100,000 children less than 16 years of age, with a prevalence of 10-17% among all cases of epilepsy diagnosed in school-aged children (Matricardi et al., 2014). CAE is also more frequently diagnosed in girls than in boys with a prevalence of 11.4% versus 2.5% (Waaler et al., 2000). There is no structural neuropathology associated with CAE, but absence epilepsy has a strong genetic component; for CAE cases, 16-45% have positive family history. The mechanism of inheritance and the genes involved in absence epilepsy are not well established, although there is evidence that CAE and other absence epilepsies are channelopathies (Crunelli and Leresche, 2002; Weber and Lerche, 2008). Large-scale genome studies have identified several susceptibility loci including the calcium channel gene and GABA receptor genes. Four mouse models of spike-wave epilepsy are caused by mutations in genes of voltage-gated calcium channels (Frankel et al., 2005). More recently, a genome wide high-density single nucleotide polymorphism (SNP)-based

linkage analysis from 41 nuclear pedigrees with at least two affected members found Trak1 variants within a susceptibility locus for CAE (Chioza et al., 2009). Bioinformatics analysis identified a Trak1 variant containing a triplet repeat in the final exon of a short Trak1 transcript, NM_014965. The repeat inserts additional glutamic acid residues in a run of glutamic acid residues. Trak1 has been shown to regulate endocytic trafficking and Trak1 has been shown to interact with the $GABA_A$ receptor α1 subunit. The altered regulation of $GABA_A$ receptors endocytic trafficking due to Trak1 mutation may be a contributing factor to pathogenesis of CAE and other types of generalized epilepsies.

Although CAE is perceived to be a benign disorder, and the majority of patients spontaneously stop having seizures during adolescence, not all children gain spontaneous remission and there still remains a need to provide children with symptomatic relief during seizure events. CAE is commonly treated with antiepileptic drugs such as ethosuximide, valproic acid, or lamotrigine, however, these drugs need to be continuously administered during the active phase of their epilepsies, are associated with attention deficits, and are only effective in about 75% of patients (Buchhalter, 2011). Moreover, neuropsychological studies have shown that patients affected by CAE have cognitive and linguistic dysfunction and behavioral disorders particularly with attention, memory, language, and reading disability, as well as hyperactivity disorder that are not improved by antiepileptic medications (Matricardi et al., 2014). Long-term studies suggest that children affected by CAE may have poor psychosocial adaptation, lower academic achievement, poor social functioning, and behavioral deficits (Wirrell et al., 1997). CAE is a complex disorder and although it is genetically determined, the mode of inheritance and precise genes involved are not understood, therefore it is important to understand the genes and susceptibility locus of this complex disorder.

1.5.3. Gastric and colorectal cancer

Gastric cancers are the fourth most common type of cancer in the world and second only to lung cancers as the leading cause of cancer death (Crew and Neugut, 2006; Orditura et al., 2014). In the year 2000, 880,000 people were diagnosed with gastric cancer and roughly 650,000 died of the disease. Gastric cancer incidence rates vary by up to 10-fold throughout the world with the highest rates in developing countries and in Asian countries such as Japan, Korea, and China. The cause of disease is multifactorial and has both genetic as well as environmental and lifestyle factors (Cancer Genome Atlas Research, 2014; El-Omar et al., 2003; Gonzalez et al., 2002), with about 10% of cases showing familial clustering (Guilford et al., 1998). The lack of early detection methods for gastric cancer is one of main reasons for poor prognosis as most patients are asymptomatic at early stages and by the time a diagnosis is reached the patients are already at advanced stages of cancer progression (Fan et al., 2005). Surgery is the only curative therapy, however, more than half of patients relapse with metastases after surgery, and the 5-year survival rate is less than 10% (Orditura et al., 2014).

In the search for effective cancer biomarkers that are capable of specific and early detection of gastric cancer, a series of monoclonal antibodies were developed using homogenates from gastric carcinoma cell line KATOIII (Fan et al., 1988). Among these antibodies one was found to immunoreact specifically with an unknown antigen present in cancerous tissues. This unknown antigen was later identified as Trak1 by means of immunoprecipitation, MALDI-TOF mass spectrometry, and bioinformatics analyses. Trak1 has hence been proposed as a novel cancer biomarker specific for gastric cancer, and colorectal subtype of gastric cancer (An et al., 2011; Thorsen et al., 2011; Zhang et al., 2009). The level of Trak1 expression was found to increase with progression of gastric carcinoma (Zhang et al., 2009). Trak1 expression is significantly higher in colorectal cancer tissues compared to normal surrounding tissues, and Trak1 is overexpressed in human colon carcinoma cell lines compared to a normal intestinal epithelial cell line (An et al., 2011). Trak1 expression significantly correlated with colorectal

cancer differentiation, invasion, and pathology and higher levels of Trak1 expression related to poorer survival outcomes in patients. The possible mechanism of Trak1 in the development of gastric cancer and colorectal cancer is unknown and Trak1 may directly play an important role in development of these cancers or may be indirectly unregulated by unknown mechanisms.

1.6. Hypotheses and organizational overview

The finding that Trak1 has a dual role in the regulation of endosome-to-lysosome trafficking and mitochondrial transport highlights the significance of Trak1 in cellular homoeostasis and organismal health. Dysfunction of Trak1 regulated processes are linked to several disease states, including hypertonia, childhood absence epilepsy, and carcinogenesis, but the pathogenic mechanisms underlying these disease states are not known. The role of Trak1 in mitochondrial transport is well established, however, whether it has a role in regulating other mitochondrial processes such as fission or fusion is not known. Proper mitochondrial dynamics are critical to cellular metabolism, calcium buffering, and regulation of apoptotic cell death through cytochrome c release. The disruption of mitochondrial dynamics is associated with a variety of human diseases, most significantly neurodegenerative diseases and cancer. Although many of the machineries directly involved in mitochondrial dynamics have been elucidated, the precise mechanisms controlling these processes remain unsolved. Furthermore, the number of disease linked proteins implicated in mitochondrial dynamics and function are increasing.

In chapter 2, I identify a novel function for Trak1 in mitochondrial fusion and provide evidence that Trak1 functionally interacts with Mitofusins to regulate mitochondrial fusion. My results reveal that depletion of Trak1 via shRNA knockdown results in fragmentation of the mitochondrial network and a decreased rate of mitochondrial fusion. Fragmented mitochondria in Trak1 depleted cells could not be rescued by the overexpression of Mitofusin proteins, suggesting a functional interaction of Trak1 with Mitofusins in mitochondrial fusion. The overexpression of Trak1 results in hyperfusion, while Mitofusin overexpression in Trak1 depleted cells fails to

induce fusion. Furthermore, in the absence of Mitofusins the overexpression of Trak1 promotes mitochondrial clustering with apposing outer mitochondrial membranes in close contact but Trak1 expression alone could not drive fusion. Taken together, my results indicate that Trak1 acts in an upstream fusion step to mediate intermitochondrial tethering, thus providing a mechanism to allow Mitofusin proteins on adjacent mitochondria to interact and drive membrane merging. Furthermore, the fragmented mitochondria in Trak1 depleted cells are more vulnerable to apoptotic cell death under nutrient starvation. My studies also reveal that the Trak1 hyrt mutation affects Trak1 targeting to mitochondria, resulting in partial mislocalization away from mitochondria to the cytosol, and impaired ability of Trak1 to promote mitochondrial fusion, revealing a novel mechanistic explanation for how the Trak1 hyrt mutation contributes to cellular dysfunction. This is the first study to identify a pre-fusion step upstream of Mitofusin in the mitochondrial fusion process as well as the first study to provide a direct link between regulation of mitochondrial transport and fusion processes.

Our lab previously identified a role for Trak1 in regulation of endosome-to-lysosome trafficking (Webber et al., 2008). Trak1 was shown to interact directly with Hrs endosomal sorting protein and was found to be required for endocytic trafficking of endocytosed EGF receptors from the early endosome to lysosomes for degradation (Weber and Lerche, 2008). A role for Trak1 in the endocytic trafficking of other proteins has not been established. The fruit fly, *Drosophila melanogaster*, contains a Trak1 orthologue named Milton. Milton is most similar in sequence homology to Trak1 but is predicted to be the functional orthlogue of GRIF-1 and HAP1 as well. In mammals, Trak1, GRIF-1, and HAP1 proteins are collectively part of the HAP1 N-terminal homologous (HAPN) domain family of proteins. The HAPN domain family of proteins has all been shown to interact with Hrs and mediate the endocytic trafficking of EGF receptors, suggesting a shared function in this family of proteins. A role for Milton in the regulation of endocytic trafficking has not been established. Milton was identified as a molecular adaptor linking mitochondria to kinesin motors and is required for transport of mitochondria to nerve

terminals. Trak1 and GRIF-1 have also been shown to have a similar function in regulating mitochondrial transport in neurons, establishing a conserved role for mitochondrial transport between flies and mammals. Milton is predicted to be the functional orthologue of all three proteins of the mammalian HAPN domain family; therefore, may have functional roles outside of mitochondrial transport and similar to Trak1, Milton could have a dual function in mitochondrial dynamics and endocytic trafficking regulation.

In chapter 3, I examine the role of Milton in *Drosophila melanogaster* development by selectively depleting Milton expression in various fly tissues using the GAL4/UAS system. In particular I use the fly eye to study endocytic trafficking defects in pigment granules. In the fly wing I examine wing vein patterning to observe possible defects in EGF receptor signaling pathways. I determined that loss of Milton in the fly eye did not affect pigment granule trafficking, which suggests that Milton may not regulate the trafficking of pigment granules. Loss of Milton in the *Drosophila* wing resulted in aberrant wing vein patterning, particularly in the expression of ectopic wing veins. These data suggest the loss of Milton in the fly wing affects MAPK signaling pathways, proving a link for Milton in regulation of EGF receptors and possibly other signaling receptors in *Drosophila* wing vein patterning. Additionally, I found the depletion of Milton in neurons results in reduced lifespan and locomotor climbing ability. Taken together my observations support a role for Milton in the normal development of various fly tissues and suggest that Milton is essential to the regulation of critical cellular processes.

In chapter 4, I describe the significance of my research as well as provide suggestions for future studies to further the understanding of Trak1 in regulation of mitochondrial dynamics and endocytic trafficking. Overall my thesis works provides new understanding about Trak1 function and the role of Trak1 in maintaining cellular homeostasis.

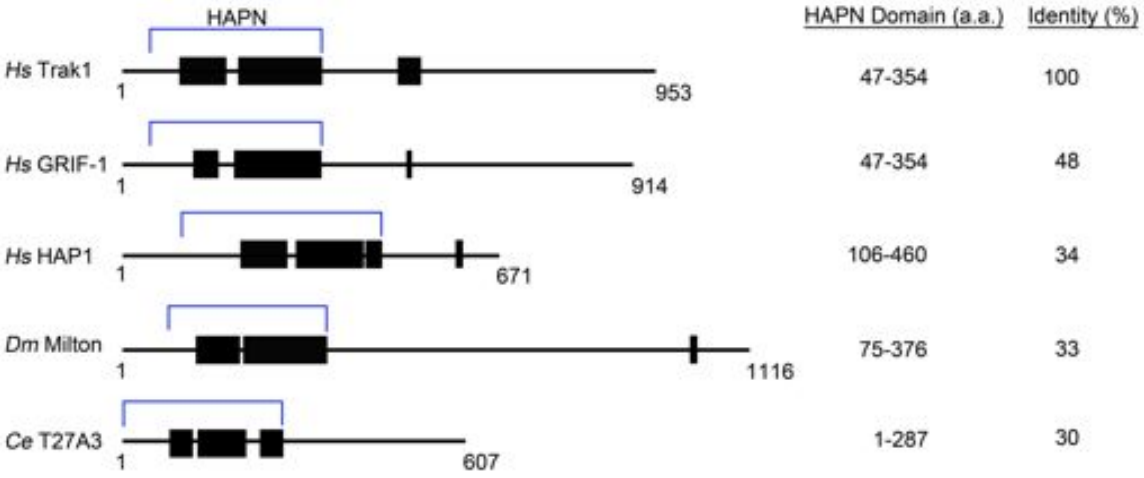


Figure 1-1. **Domain structure of Trak1 and other HAPN domain family of proteins.** All HAPN domain family proteins contain three predicted coiled-coil domains shown in black boxes. The coiled-coil regions are as follows: *Hs* Trak1: 104-186, 207-356, 492-532; *Hs* GRIF-1: 126-170, 198-354, 507-519; *Hs* HAP1: 212-293, 307-427, 431-460; 593-606; *Dm* Milton: 133-209, 226-377, 1021-1034; *Ce* T27A3: 84-128, 141-226, 245-289. The location of the HAPN domain of each protein and the amino acid identity of each protein relative to the protein sequence of *Hs* Trak1 are indicated. Abbreviations: *Hs*, *Homo sapiens*; *Dm*, *Drosophila melanogaster*; *Ce*, *Caenorhabditis elegans*; Trak1, trafficking protein, kinesin-binding 1; GRIF-1, $GABA_A$ receptor interacting factor-1; HAP1, huntingtin-associated protein 1.

Protein	**Function**	**HAPN domain Protein & Binding Region**	**Reference**
Hrs	Early endosome sorting	-Trak1 (a.a. 354-953, 354-507 predicated based on 50% a.a. similarity to GRIF-1) -GRIF-1 (a.a. 359-507) -HAP1 (a.a. 246-425)	-Webber et al., 2008 -Kirk et al., 2006 -Li et al., 2002
$GABA_A$ Receptor	Cell surface receptor	-Trak1 (a.a. ?) -GRIF-1 (a.a. 124-283) -HAP1 (a.a. 220-520)	-Gilbert et al., 2006 -Beck et al., 2002 -Kittler et al., 2004
Miro	Mitochondrial transport	-Trak1 (a.a. 599-800) -Milton (a.a. 750-1,116) -GRIF-1 (a.a. 476-700)	-Koutsopoulos et al., 2010 -Glater et al., 2006 -MacAskill et al., 2009
KHC	Anterograde microtubule motor	-Trak1 (a.a. 201-306) -Milton (a.a. 1-450) -GRIF-1 (a.a. 124-283) -HAP1 (a.a. 153-320)	-Smith et al., 2006 -Stowers et al., 2002 -Brickley et al., 2005 -Twelvetrees et al., 2010
KLC	Anterograde microtubule motor	-HAP1 (a.a. 445-599)	-McGuire et al., 2006
OGT	Enzyme that catalyzes O-GlcNAc addition to Ser and Thr	-Trak1 (a.a. 639-859) -Milton (a.a. 450-750) -GRIF-1 (a.a. 359-507)	-Iyer et al., 2003; Iyer and Hart, 2003 -Pekkurnaz et al., 2014 -Iyer et al., 2003
$P150^{GLUED}$	Component of Dynactin/Dynein retrograde microtubule motor	-Trak1 (2 binding regions: a.a. 1-360 and 396-953) -GRIF-1 (a.a. ?) -HAP1 (a.a. 287-445)	-van Spronsen et al., 2013 -van Spronsen et al., 2013 -Li et al., 1998
Kir2.1	Cell surface potassium channel	-GRIF-1 (a.a. 1-497)	-Grishin et al., 2006
IP3R	Calcium channel	-HAP1 (a.a. 273-599)	-Tang et al., 2003
Huntingtin	Scaffold protein	-HAP1 (a.a. 278-370)	-Li et al., 1998
DISC1	Scaffold protein	-Trak1 (a.a. ?)	-Ogawa et al, 2014
Trak1	Endocytic trafficking, mitochondrial transport	-Trak1 (a.a. 1-380) -GRIF-1 (a.a. ?)	-Koutsopoulos et al., 2010 -Koutsopoulos et al., 2010
GRIF-1	Endocytic trafficking, mitochondrial transport	-Trak1 (a.a. ?) -GRIF-1 (283-913)	-Koutsopoulos et al., 2010 -Brickley and Stephenson, 2011
EGFR	Cell surface receptor	-HAP1 (a.a. ?)	-Li et al., 2002
Trk A	Cell surface receptor	-HAP1 (a.a. ?)	-Rong et al., 2006
BDNF	Growth factor	-HAP1 (a.a. ?)	-Gauthier et al., 2004

Table 1-1. **Proteins that interact with HAPN domain family of proteins.** Abbreviations: Trak1, trafficking protein, kinesin-binding 1; GRIF-1, $GABA_A$ receptor interacting factor-1; HAP1, huntingtin-associated protein 1; Hrs, hepatocyte growth factor-regulated tyrosine kinase substrate; $GABA_A$, γ-aminobutyric acid type A; Miro, Mitochondrial Rho GTPase; KHC, kinesin heavy chain; HLC, kinesin light chain; OGT, O-linked β-N-acetylglucosamine transferase; Kir2.1, inward-rectifying potassium ion channel; IP3R, inositol 1,4,5-triphosphate receptor; DISC1, disrupted in schizophrenia 1; EGFR, epidermal growth factor receptor; Trk A, Tropomyosin receptor kinase A; BDNF, Brain-derived neurotrophic factor.

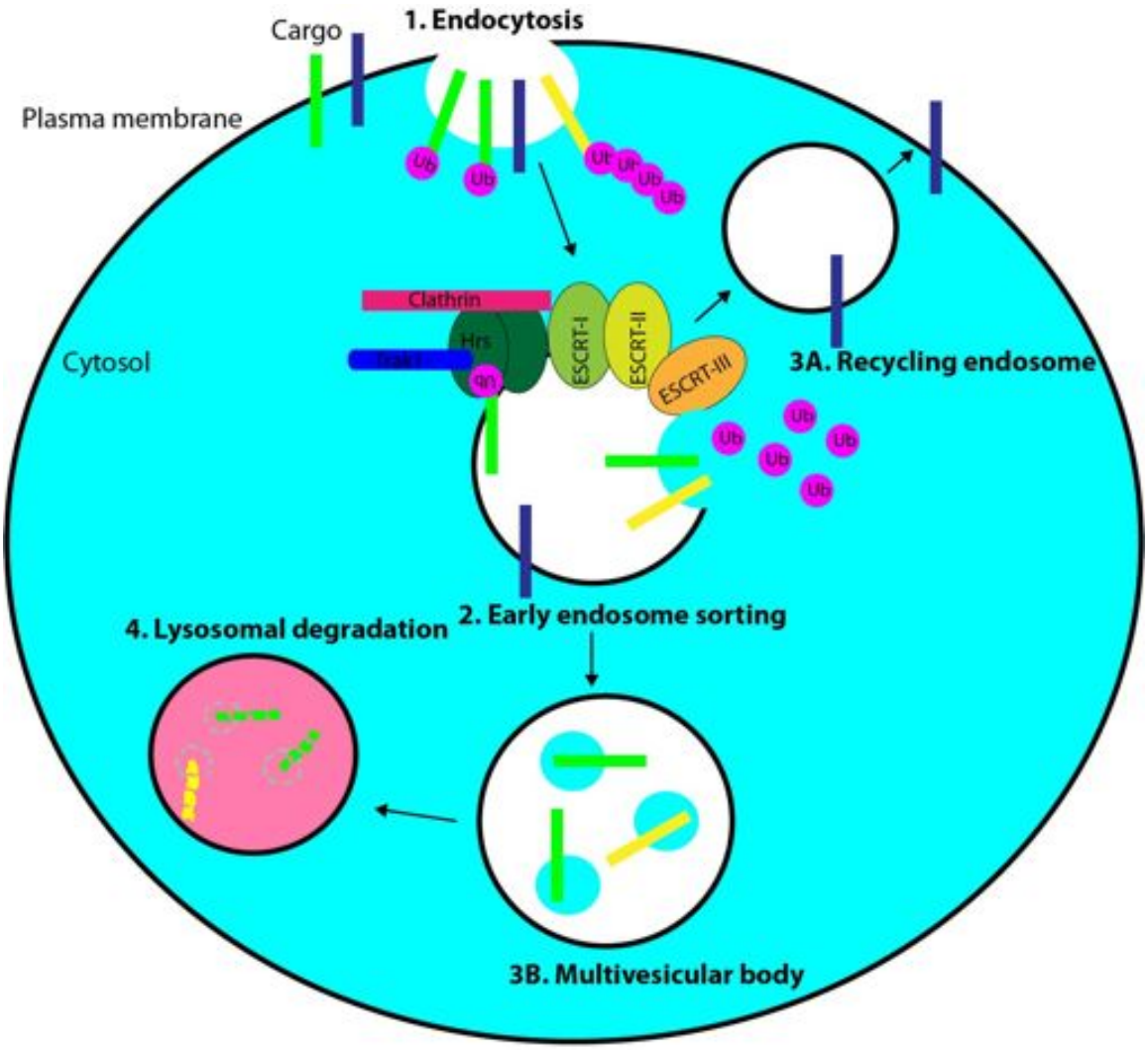


Figure 1-2. **The endocytic trafficking pathway.** Receptors at the plasma membrane are endocytosed constitutively or in response to ligand binding (1). Endocytosis is mediated by both ubiquitin-dependent and independent mechanisms. The endocytosed cargo proteins are subsequently trafficked to the early endosome (2). At the early endosome cargo receptors are either sorted to the recycling pathway which returns the cargo back to the plasma membrane (3A) or sorted to the lysosomal degradation pathway (3B and 4). Ubiquitinated cargo proteins are recognized by Hrs, which contains a ubiquitin-interacting domain. Hrs is enriched in regions of the early endosome containing a specialized bilayer of clathrin. Trak1 directly binds Hrs and is

required for the endosome to lysosome trafficking of internalized EGF (epidermal growth factor) receptors. Hrs also recruits the ESCRT complexes I, II, and III, which removes the ubiquitin molecules from the cargo proteins and incorporates the cargo proteins into intraluminal vesicles of multivesicular bodies for transport to lysosomes (3B). Multivesicular bodies eventually fuse with lysosomes, where the cargoes are then degraded by lysosomal hydrolases (4). Abbreviations: Ub, Ubiquitin; Hrs, hepatocyte growth factor-regulated tyrosine kinase substrate; ESCRT, Endosomal Sorting Complexes Required for Transport; Trak1, trafficking protein, kinesin-binding 1.

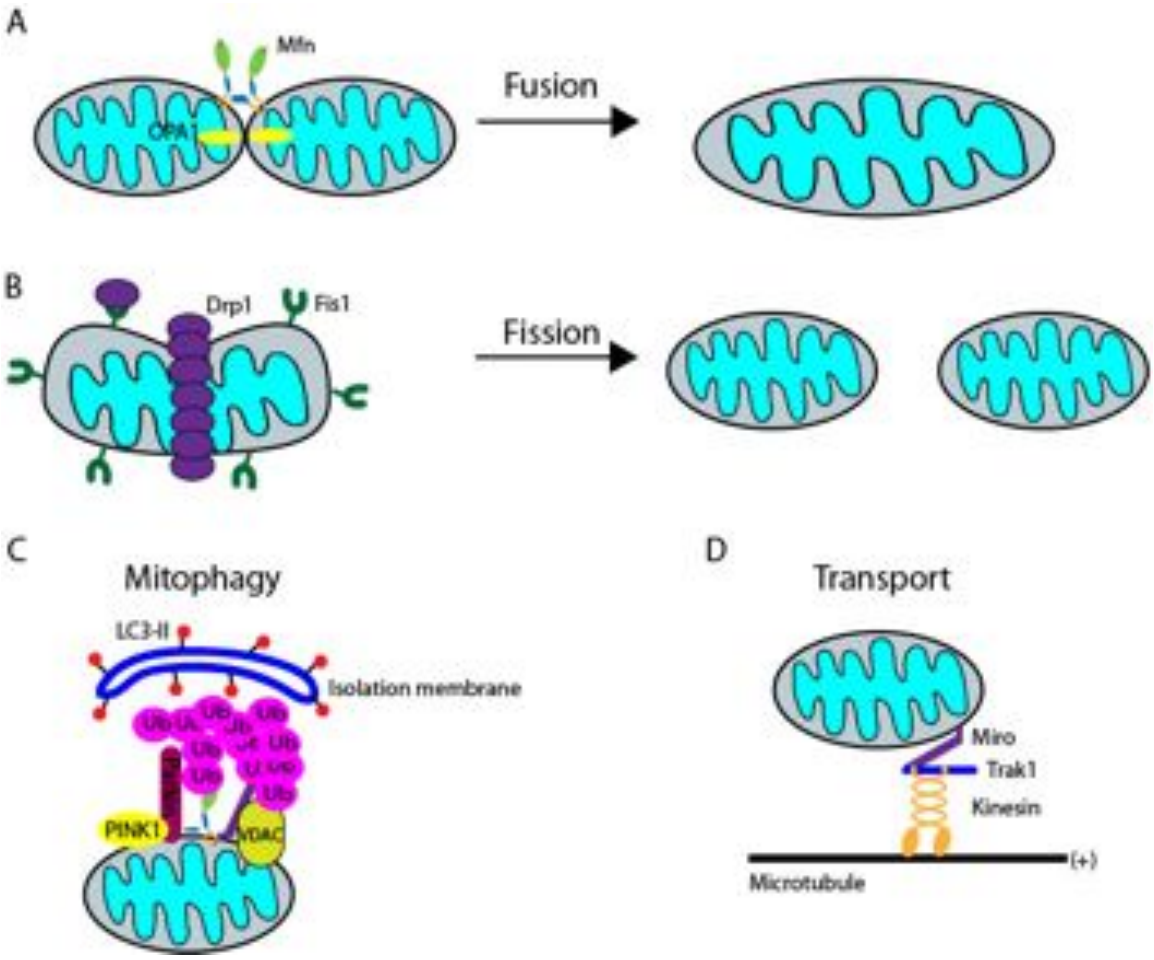


Figure 1-3. **Molecules that regulate mitochondrial dynamics.** (A) Mitochondria have two membranes that act in concert to regulate fusion. Mfn (Mfn1 or Mfn2) and OPA1 regulate mitochondrial outer and inner membrane fusion, respectively. Mfn is an outer mitochondrial membrane (OMM) protein with a cytosolic N-terminal GTPase domain (lime green oval) and two coiled-coil regions (light blue ovals). The C-terminal coiled-coil region forms antiparallel oligomers between Mfns on apposing mitochondria, while GTPase activity drives membrane merger. OPA1 is an inner mitochondrial membrane (IMM) GTPase that is required for IMM fusion. (B) Drp1 and Fis1 are required for mitochondrial fission. Fis1 is an integral protein that is uniformly localized to the OMM and recruits Drp1 to the OMM from the cytosol. During fission, Drp1 oligomerizes to form a ring around the circumference of the OMM that constricts to divide

the mitochondrion. (C) Mitophagy is required to maintain mitochondria quality control. In mammals, mitophagy of damaged mitochondria is regulated by parkin and PINK1. During mitophagy, parkin an E3 ubiquitin ligase ubiquitinates several OMM proteins, including Mfns, Miros, and VDAC to trigger their degradation and to signal formation of the autophagosome isolation membrane, which is marked by LC3-II proteins. (D) The key conserved and major mitochondrial anterograde transport machinery is the Miro/Trak1 complex. Miro (Miro 1 or Miro 2) is a transmembrane OMM GTPase with two EF-hand Ca^{2+}-binding domains. Trak1 is recruited to mitochondria through interaction with Miro. Trak1 also binds kinesin motors to facilitate the transport of mitochondria along microtubule tracks. Abbreviations: OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane; Mfn, Mitofusin; OPA1, optic atrophy type 1; Fis1, fission 1; Drp1, dynamin-related protein 1; PINK1, PTEN-induced putative kinase protein 1; VDAC, voltage-dependent anion channel; Ub, Ubiquitin; Trak1, trafficking protein, kinesin-binding 1; LC3-II, Microtubule-associated protein 1A/1B-light chain 3-II.

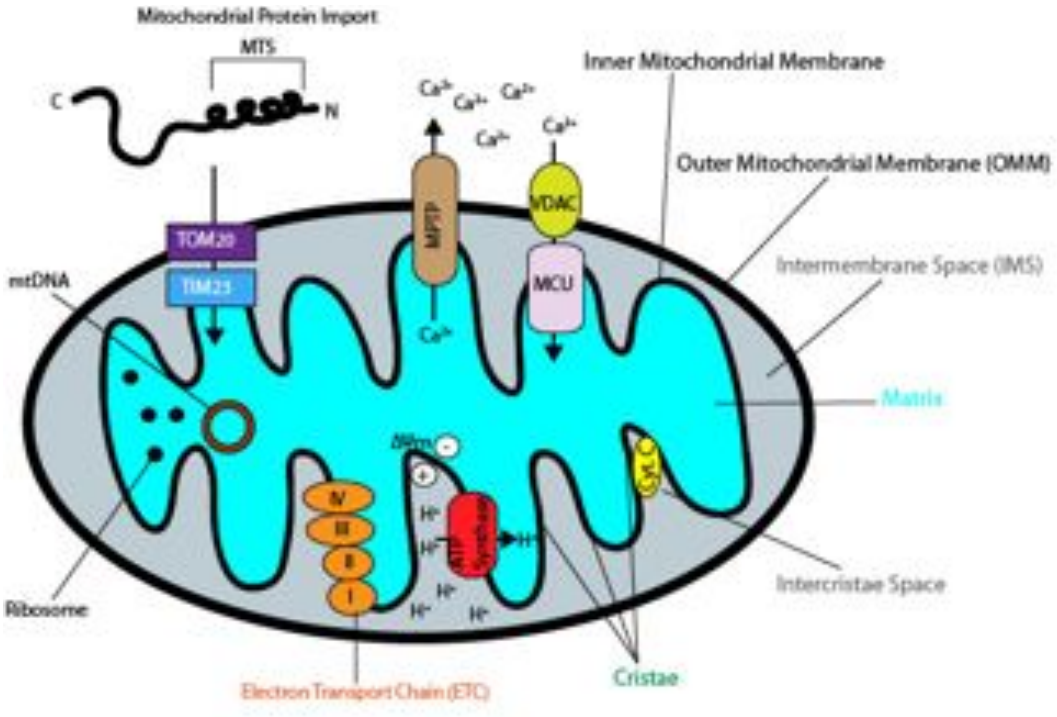


Figure 1-4. **Structure of mitochondria.** Mitochondria are essential organelles that are responsible for generating ATP, calcium buffering, and regulation of apoptosis. Mitochondria are organized into several sub-compartments that are critical to its function. Mitochondria contain two separate membrane layers, the outer mitochondrial membrane (OMM) and the inner mitochondrial membrane (IMM), which separate the two aqueous compartments, the intermembrane space (IMS) and the matrix. Proteins with mitochondrial targeting sequences (MTS) are imported into the mitochondrial matrix through the membrane pore proteins, TOM20 and TIM23. The electron transport chain machinery (I-IV) is located on IMM folds called cristae. The ETC generates a negative membrane potential ($\Delta\Psi_m$) by pumping H^+ into the IMS against the concentration gradient, while H^+ is pumped back into the matrix by ATP synthase to generate ATP. Cytochrome c is located in the intercristae space, the region between the cristae folds. Import of Ca^{2+} into the mitochondrial matrix occurs through VDAC and MCU channels while Ca^{2+} release occurs through MPTP channels. Mitochondria have their own circular genome (mtDNA) and ribosomes located in the matrix. Abbreviations: H^+, hydrogen ions; Ca^{2+}, calcium

ions; MTS, mitochondrial targeting sequence; Cyt. c, cytochrome c; MCU, mitochondrial calcium uniporter; ATP, adenosine triphosphate; VDAC, voltage-dependent anion channel; MPTP, mitochondrial permeability transition pore; TOM20, translocase of the outer membrane; TIM23, translocase of the inner membrane; $\Delta\Psi_{m,}$ mitochondrial membrane potential.

Chapter 2:

Hypertonia-linked protein Trak1 is a novel regulator of mitochondrial fusion

2.1. Abstract

Proper mitochondrial dynamics are critical for cellular metabolism, calcium buffering, and regulation of apoptotic cell death, and disruption of these cellular processes is associated with a variety of diseases including neurodegenerative diseases and cancer. A truncation mutation in Trak1 (Trak1 hyrt) was found to cause hypertonia in mice. In humans, elevated levels of Trak1 has been linked to carcinogenesis, and a Trak1 variant has been associated to childhood absence epilepsy, highlighting the importance of understanding Trak1's function. Although several proteins are found to regulate mitochondrial dynamics the precise mechanisms remain largely unknown. The function of Trak1 in mitochondrial transport is well established however, whether it has a role in regulating other mitochondrial processes is not known. Here, we report that Trak1 is essential for maintaining proper mitochondrial morphology and is necessary for mitochondrial fusion. We found that depletion of Trak1 inhibits mitochondrial fusion and leads to mitochondrial fragmentation. Fragmented mitochondria in Trak1 depleted cells could not be rescued by the overexpression of Mitofusin proteins, suggesting a functional interaction of Trak1 with Mitofusins in mitochondrial fusion. Furthermore, the fragmented mitochondria in Trak1 depleted cells are more vulnerable to apoptotic cell death under nutrient starvation. In contrast, cells overexpressing Trak1 promotes mitochondrial hyperfusion leading to abnormally elongated and enlarged mitochondria, consistent with elevated levels of fusion. Additionally, our results showed that the Trak1 hyrt mutation disrupts the mitochondrial localization of Trak1 and its regulation of mitochondrial fusion, suggesting a pathogenic loss-of-function mechanism for this mutation. These findings reveal a novel function of Trak1 in mitochondrial fusion and indicate Trak1 as an essential protein for maintaining mitochondrial health and cell survival.

2.2. Introduction

Mitochondria are essential organelles that are critical for cellular energy production, calcium buffering, and regulation of apoptotic cell death. Mitochondria form an interconnected network that is highly dynamic, constantly undergoing fusion, fission, transport and turnover. Cycles of fusion and fission are normally balanced to maintain the steady-state morphology of mitochondria (Twig et al., 2008; Wang et al., 2012). Mitochondrial fusion and fission are controlled by the opposing actions of different dynamin family of GTPases. The outer mitochondrial membrane GTPases Mitofusin 1 and 2 (Mfn1 and Mfn2) and the inner mitochondrial membrane GTPase OPA1 are essential for outer and inner mitochondrial membrane fusion, respectively (Chen et al., 2003a; Cipolat et al., 2004; Legros et al., 2002; Santel and Fuller, 2001). Dyamin related protein (Drp1) is essential to mitochondrial fission (Smirnova et al., 2001). Fusion is believed to be a cell protective mechanism allowing mitochondria to mix their contents and can lead to increase mitochondrial respiratory capacity (Tondera et al., 2009). For example, during starvation and cellular stress, mitochondria elongate and hyperfuse to sustain ATP production and prevent apoptotic cell death, in a process termed stress-induced mitochondrial hyperfusion (Gomes et al., 2011; Rambold et al., 2011; Tondera et al., 2009). Fission, on the other hand, allows for equal segregation of mitochondria during cell division (Smirnova et al., 2001), and is required for apoptosis (Frank et al., 2001). Fragmented mitochondria are also linked to mitochondrial dysfunction and neuronal cell death and may contribute to pathogenesis of neurodegenerative disorders (Cho et al., 2010; Knott et al., 2008; Reddy and Shirendeb, 2012; Wang et al., 2008). The dynamic properties of mitochondria are essential to its function and to overall cellular health (Chan, 2012). Defects in machinery regulating mitochondrial dynamics are implicated in a wide-range of human diseases, including inherited and age-associated neurodegenerative disorders (Chen and Chan, 2009; Winklhofer and Haass, 2010; Zuchner et al., 2004), aging (Crane et al., 2010; Seo et al., 2010), and cancer (Rehman et al., 2012; Zhao et al., 2013a).

Trafficking protein, kinesin-binding 1 (Trak1) is a ubiquitously expressed protein functioning in mitochondrial transport (Brickley and Stephenson, 2011; Stowers et al., 2002; van Spronsen et al., 2013) and endosome-to-lysosome trafficking (Webber et al., 2008). For mitochondrial transport, Trak1 acts as an adaptor protein by binding the mitochondrial outer membrane Rho GTPase Miro and kinesin heavy chains to facilitate transport of mitochondria on microtubules (Brickley and Stephenson, 2011; Stowers et al., 2002), however in this present study we identify a novel function of Trak1 in mitochondrial fusion independent of its role in mitochondrial transport. Mutations in Trak1 have been directly linked to neurological disorders (Chioza et al., 2009; Gilbert et al., 2006). A frameshift mutation in the *Trak1* gene causes recessively transmitted hypertonia in mice (Gilbert et al., 2006). Hypertonia is a movement pathology characterized by stiff gait, abnormal posture, jerky movements, and tremor, and is associated with several neurodegenerative disorders, including cerebral palsy, dystonia, Parkinson's disease, stroke, and epilepsy. A *Trak1* variant has also been linked to childhood absence epilepsy in humans (Chioza et al., 2009). Furthermore, elevated levels of Trak1 are reported in gastric and colorectal cancers (An et al., 2011; Zhang et al., 2009). The mechanism by which altered Trak1 function contributes to disease states is not known.

In this study, we identify a novel function for Trak1 in mitochondrial fusion and found that the hypertonia-linked Trak1 mutation (Trak1 hyrt) disrupts the mitochondrial localization of Trak1 and its regulation of mitochondrial fusion. Our results reveal that loss of Trak1 via shRNA knockdown results in fragmentation of the mitochondrial network and a decreased rate of mitochondrial fusion. Trak1 depletion also inhibits stress-induced mitochondrial hyperfusion and leads to apoptotic cell death under stress conditions. Fragmented mitochondria resulting from *Trak1* gene silencing could not be rescued by overexpression of Mitofusin proteins, suggesting that Trak1 is a necessary component of the mitochondrial fusion machinery. Furthermore, we found that Trak1 hyrt mutation exhibited partially disrupted targeting to mitochondria and

displayed limited capability in regulating mitochondrial fusion, suggesting a novel mechanism in hypertonia pathogenesis. Our findings provide novel insights into the essential contribution of Trak1 in normal mitochondrial physiology and reveal Trak1 to be a novel regulator of mitochondrial fusion.

2.3. Results

2.3.1. Depletion of endogenous Trak1 causes mitochondrial fragmentation

To assess the role of Trak1 on mitochondrial dynamics, we generated stably transfected HeLa cells expressing Trak1-targeting shRNAs to deplete the endogenous Trak1 proteins. Two independent clonal Trak1 shRNA lines (shTrak1-1 and shTrak1-2) were generated and both demonstrated a high level of Trak1 protein depletion by western blot, compared to the non-targeting control shRNA (shCTRL) (Fig. 2-1 A). Our immunofluorescence confocal microscopic analyses revealed that in shTrak1 cells, mitochondrial morphology and distribution as visualized by MitoTracker staining were severely altered, resulting in both mitochondrial fragmentation and loss of mitochondria at the cell periphery with concomitant clustering of mitochondria near the nucleus (Fig. 2-1 B-D). These results suggest that loss of Trak1 results in both defects to mitochondrial transport as well as fusion/fission dynamics. Mitochondria appear small and spherical in shTrak1 cells compared to the tubular mitochondria prominent in control cells (Fig. 2-1 B, C).

Individual mitochondria cannot be resolved by confocal microscopy; therefore, super-resolution imaging analysis using 3D-structured illumination microscopy (3D-SIM) (Huang et al., 2009; Schermelleh et al., 2008) was preformed to visualize and measure mitochondria identified by mitochondrial markers, including transfected DsRed-mito, and antibodies against endogenous TOM20 (Fig. 2-1 E). In shTrak1 cells spherical mitochondria and fragmented tubular mitochondria were observed. In comparison, mitochondria in control cells were long and tubular.

Quantitative analyses of mitochondrial size revealed a nearly two-fold decrease in mitochondrial length and area in shTrak1 cells compared to control (Fig. 2-1 F, G).

Morphological abnormalities in mitochondria are often accompanied by pathological changes in mitochondrial ultrastructure (Chung and Suh, 2002; Stichel et al., 2007; Zick et al., 2009). To determine whether the mitochondrial fragmentation seen in shTrak1 cells is associated with ultrastructural changes in mitochondria we performed transmission electron microscopy (TEM) analyses to assess mitochondria and cristae morphology (Fig. 2-1 H-L). Mitochondria from shCTRL cells were tubular in appearance and displayed well-defined outer and inner mitochondrial membrane structures, including parallel, evenly distributed cristae membranes. In contrast, mitochondria from shTrak1 cells were predominately round and spherical (Fig. 2-1 H), with shorter cristae membranes and an overall reduction in cristae membranes compared to mitochondrial membrane (Fig. 2-1 K, L). In accord with our 3D-SIM microscopy findings, we found overall decreased mitochondrial size in shTrak1 cells compared to control using TEM. However, we found that mitochondrial length and area (Fig. 2-1 I, J) were notably smaller than those measured under 3D-SIM microscopy (Fig. 2-1 F, G), which is likely due to the higher resolution of electron microscopy allowing for a more accurate measurement of individual mitochondria.

2.3.2. Trak1 functions in regulation of mitochondrial fusion

Mitochondrial fragmentation can result from elevated fission or inhibition of fusion. Given that Trak1 depletion results in mitochondrial fragmentation, we next examined the possibility that Trak1 directly effects mitochondrial fusion. To measure fusion and fission events we used time-lapse live cell confocal microscopy to track a population of mito-Dendra2 labeled mitochondria for 20 minutes (Fig. 2-2). Mito-Dendra2 within a defined region of interest (ROI) was

photoconverted from green to red at time 0 and the colocalization of red fluorescent mitochondria overlapping with green fluorescent mitochondria was used to approximate the rate of fusion over time. Quantitative analyses indicated that the rate of fusion was reduced by greater than 2-fold in shTrak1 cells compared to control (Fig 2-2 B). These data suggest that Trak1 plays a role in mitochondrial fusion, and that the mitochondrial fragmentation resulting from Trak1 depletion occurs due to reduced fusion rather than increased fission.

2.3.3. Hypertonia-linked mutation impairs Trak1 mitochondrial localization and function

Next, to assess the ability of Trak1 in rescuing the mitochondrial defects seen in shTrak1 cells, experiments were performed with Trak1 WT and hypertonia-associated Trak1 mutant (Trak1 hyrt) shRNA-resistant rescue constructs (Fig. 2-3). Trak1 hyrt mutation confers recessively transmitted hypertonia in mice, and represents a frameshift mutation in the mouse *Trak1* gene, resulting in a 115 amino acids C-terminal protein truncation (Fig. 2-3 A, B) (Gilbert et al., 2006; Webber et al., 2008). Rescue of shTrak1 cells with full-length Trak1 WT restored normal mitochondrial morphology and cellular distribution of mitochondria (similar to mitochondria of control cells, shCTRL), while rescue with Trak1 hyrt only partially restored normal mitochondrial morphology and cellular distribution (Fig. 2-3 C-E). Protein expression of transfected rescue constructs in shTrak1 cells were near endogenous Trak1 levels seen in control cells as shown by western blot (Fig. 2-3 B), indicating that the inability of Trak1 hyrt to fully rescue mitochondrial morphology is due to a partial loss of function.

Although the Trak1 hyrt mutation is not expected to interfere with mitochondrial localization, as the missing amino acids are outside of the predicated mitochondrial-targeting domain (Koutsopoulos et al., 2010) and our microscopy analyses showed co-localization of Trak1 hyrt with mitochondrial markers (Fig. 2-3 C), we also observed an increase in the cytosolic level of Trak1 hyrt compared to Trak1 WT and an inability to fully rescue mitochondrial abnormalities

in shTrak1 cells (Fig. 2-3 D,E). To examine the possibility that Trak1 hyrt has altered localization to mitochondria we performed subcellular fractionation assays to assess the relative cytosolic and mitochondrial distributions of Trak1 proteins (Fig. 2-4). Endogenous Trak1 and exogenously expressed GFP-tagged Trak1 WT display a similar distribution profile of Trak1 protein in the cytosolic and mitochondrial fractions, while GFP-Trak1 hyrt protein is more prominently found in the cytosolic fraction with a significantly smaller pool found in the mitochondrial fraction (Fig. 2-4 C-D). The impaired targeting of Trak1 hyrt to mitochondria may cause a partial loss of mitochondrial function, which is consistent with our finding that Trak1 hyrt could not fully rescue mitochondria morphology in shTrak1 cells. These data suggest that Trak1 hyrt mutation impairs mitochondrial targeting and that the impaired targeting may, in part, disrupt mitochondrial fusion and contribute to hypertonia pathogenesis.

2.3.4. Trak1 overexpression induces mitochondrial hyperfusion

Given that Trak1 depletion results in mitochondrial fragmentation and reduced fusion activity, we next examined the possibility that increasing Trak1 protein levels promotes mitochondrial fusion. Immunofluorescence confocal microscopy analyses indicated that overexpression of GFP-tagged Trak1 WT results in mitochondria that are hyperfused, which is characterized by abnormally elongated and enlarged mitochondria (Fig. 2-5). For comparison, cells overexpressing GFP vector alone displayed normal mitochondrial morphology (Fig. 2-5 B-D). In Trak1 WT overexpressing cells we also observed a population of mitochondria highly concentrated at the cellular periphery (Fig. 2-5 B arrowhead). Taken together, our data suggest that Trak1 overexpression increases the rates of fusion, resulting in mitochondria with a hyperfused phenotype.

To test the possibility that the Trak1 hyrt mutation confers mitochondrial pathology we assessed the effect of Trak1 hyrt expression on mitochondrial fusion. We overexpressed Trak1

hyrt protein or Trak1 WT protein at similar levels (Fig. 2-5 A) and performed quantitative analysis on mitochondrial morphology (Fig. 2-5 C, D). Our results indicate that although a population of Trak1 hyrt is targeted to mitochondria, it is less effective at inducing mitochondrial hyperfusion. Compared to Trak1 WT, Trak1 hyrt has an intermediate effect on mitochondrial fusion, with only 59.48% ± 8.57% of transfected cells exhibiting hyperfused mitochondria, compared to 91.03% ± 4.92% in Trak1 WT overexpressing cells. Additionally, cells expressing Trak1 hyrt predominately displayed elongated mitochondria rather than enlarged mitochondria seen in Trak1 WT (Fig. 2-5 D). In a small number of cells expressing Trak1 hyrt enlarged mitochondria were observed, but they were much smaller and fewer in number compared to cells overexpressing Trak1 WT. The enlarged mitochondria may represent a stronger hyperfusion state than elongated mitochondria and perhaps as mitochondria initially start to hyperfuse they appear elongated and then collapse into an enlarged structure as fusion continues.

To assess the mitochondrial localization of Trak1 WT or hyrt at high resolution, we performed 3D-SIM analyses of cells overexpressing Trak1 proteins. By SIM microscopy both Trak1 WT and Trak1 hyrt proteins can be clearly seen decorating the outer mitochondrial membrane (OMM) and co-localizing with the OMM marker TOM20, but not with the matrix marker DsRed-mito (Fig. 2-6 A). However, Trak1 hyrt exhibits an increase in cytosolic staining compared to Trak1 WT. Cells expressing GFP vector alone did not have altered mitochondrial morphology; GFP is cytosolic, and does not co-localize with mitochondrial proteins (Fig. 2-6 A). Quantification of mitochondrial size indicates a significant increase in mitochondrial length (Fig. 2-6 B) and mitochondrial width (Fig. 2-6 C) when Trak1 WT or Trak1 hyrt is overexpressed compared to GFP control. However, consistent with our confocal results overexpression of Trak1 hyrt largely promotes an increase in mitochondrial length (elongated mitochondria) while overexpression of Trak1 WT largely promotes an in mitochondrial width (enlarged mitochondria).

We next performed TEM analyses to determine whether the mitochondrial hyperfusion seen in Trak1 overexpressing cells produces ultrastructural abnormalities (Fig. 2-7). Normal mitochondrial morphology was observed in mock-transfected control cells, while Trak1 WT overexpressing cells displayed both abnormally enlarged spherical mitochondria and abnormally long tubular mitochondria. In Trak1 hyrt expressing cells we detected only abnormally long tubular mitochondria (Fig. 2-7). Consistent with our SIM findings, quantification of mitochondrial size indicates a significant increase in mitochondrial length (Fig. 2-7 B) and mitochondrial width (Fig. 2-7 C) when Trak1 WT or Trak1 hyrt is overexpressed compared to mock transfected cells. However, compared to Trak1 hyrt overexpression, the overexpression of Trak1 WT results in mitochondria with increased mitochondrial width distribution (Fig. 2-7 C), while compared to Trak1 WT overexpression, the overexpression of Trak1 hyrt results in mitochondria with increased mitochondrial length distribution (Fig. 2-7 B). Cells overexpressing Trak1 WT or Trak1 hyrt displayed disrupted cristae organization in which the cristae membranes appeared curled and generally disorganized, especially in enlarged mitochondria (Fig. 2-7 A). Quantification of cristae morphology revealed an increase in cristae length in Trak1 WT overexpressing cells (Fig. 7 D) due to enlarged mitochondria, which have greatly increased mitochondrial width and consequently longer cristae but not a significant increase in the ratio of cristae membrane to outer mitochondria membrane (OMM) (Fig. 7 E).

2.3.5. Trak1 functions upstream of Mitofusins in regulating mitochondrial fusion

In mammals there are two GTPases, Mitofusins Mfn 1 and Mfn 2, located on the OMM that are essential to mitochondrial membrane fusion (Eura et al., 2003; Legros et al., 2002). The Mitofusin proteins directly participate in mitochondrial fusion and overexpression of either Mfn1 or Mfn2 stimulates mitochondrial fusion, resulting in mitochondria with elongated hyperfused morphology (Legros et al., 2002; Rojo et al., 2002). We tested the possibility that enhanced

expression of Mitofusin proteins can reverse the mitochondrial fragmentation observed in shTrak1 cells (Fig. 2-8). Our results demonstrate that overexpression of Myc-tagged Mfn1 or Mfn2 in shCTRL cells are sufficient to drive mitochondrial hyperfusion, resulting in mitochondria that are elongated with increased connectivity, even at the cell periphery (Fig. 2-8 A, C). More importantly, in Trak1 depleted cells overexpression of Mfn1 or Mfn2 could not compensate for Trak1 loss, and the mitochondrial network remained fragmented, similar to untransfected shTrak1 cells (Fig. 2-8 B, C), suggesting a functional interaction of Trak1 with Mitofusins in mitochondrial fusion, and that Trak1 acts upstream of Mitofusins in mitochondrial fusion.

The mitochondrial Rho GTPases Miro1 and Miro2 are core components of the mitochondrial trafficking machinery and have been shown to enhance movement of mitochondria when overexpressed (Macaskill et al., 2009b; Saotome et al., 2008). Interestingly, Miro1 and Miro2 overexpression has also been shown to increase mitochondrial length (Fransson et al., 2006; Saotome et al., 2008). Because of our observation that Trak1 depletion results in loss of peripheral mitochondria (Fig. 2-1 B, D) we tested whether overexpression of Miro1 or Miro2 could promote mitochondria transport to restore normal mitochondrial distribution or to promote fusion to restore the fragmentation defects observed in shTrak1 cells (Fig. 2-8 B-D). We found that overexpression of Myc-tagged Miro1 or Miro2 in shCTRL cells resulted in hyperfused mitochondria that were highly elongated (Fig. 2-8 A, C). However, in shTrak1 cells, mitochondria remained perinuclear and fragmented following Miro1 or Miro2 overexpression, indicating that Miro proteins could not rescue shTrak1-induced mitochondria transport defects or mitochondrial fragmentation (Figure 2-8 B-D).

2.3.6. Trak1 overexpression promotes mitochondria tethering in Mitofusin-deficient cells

To determine if Trak1 alone could promote mitochondrial fusion in the absence of Mitofusin proteins, we use mouse embryonic fibroblasts (MEFs) with genetic deletions of both Mfn1 and Mfn2 ($MFN^{-/-}$ MEF) (Chen et al., 2005). Because these cells lack Mitofusin activity, their mitochondria are highly fragmented and therefore can be used to assess fusion-promoting factors that bypass Mitofusins to generate tubular mitochondria. In wild-type (WT) MEFs the exogenous expression of the profusion proteins Mfn1, Mfn2, Miro1, Miro2, and Trak1 promoted mitochondrial hyperfusion (elongation) (Fig. 2-9 A, B), similar to our results obtained from HeLa cells (Fig 2-8 A, C and Fig. 2-5 B, C). Additionally, in WT MEFs the overexpression of Trak1 hyrt resulted in an intermediate hyperfusion effect, compared to Trak1 WT overexpression (Fig. 2-9 A, B). In $MFN^{-/-}$ MEFs expression of Mfn1 or Mfn2 fully rescued mitochondrial fragmentation to a normal tubular morphology (Fig. 2-9 C, D). However, the overexpression of Trak1 could not compensate for loss of Mitofusin proteins and mitochondria remain fragmented (Fig. 2-9 C, D). Interestingly, the overexpression of Trak1 resulted in extensive clustering and aggregation of mitochondria (Fig 2-9 C, E). Trak1 hyrt was less effective at inducing mitochondrial aggregation, consistent with the moderate effects of this mutation on mitochondrial fusion. Mitochondrial clustering or fusion is not seen with overexpression of Miro proteins (Fig. 2-9 C-E), suggesting that mitochondrial clustering is specifically induced by Trak1 in MFN deficient cells.

Imaging of these cells under higher magnification using TEM shows that overexpression of Trak1 WT in $MFN^{-/-}$ cells resulted in highly clustered mitochondria with apposing outer mitochondrial membranes in close contact (Fig. 2-10 A, B), but did not promote mitochondrial fusion (Fig. 2-10 C, D). TEM analysis of Trak1 hyrt overexpression in $MFN^{-/-}$ MEF show reduced clustering with a majority of mitochondria being spatially separated. Quantification of the distance between apposing mitochondria indicates that a majority of cells overexpressing

Trak1 WT are less than 30 nm apart, compared to mock-transfected cells, which do to show mitochondrial membranes in close juxtaposition (Fig. 2-10 B). Trak1 hyrt overexpression has a moderate effect on clustering (Fig. 2-10 B). These results suggest that Trak1 promotes an upstream event prior to Mitofusin activity in which Trak1 promotes mitochondrial tethering, prior to the membrane fusion event directed by Mitofusin proteins.

2.3.7. Trak1 is essential for stress-induced mitochondrial hyperfusion and pro-survival response

During nutrient starvation mitochondrial elongation occurs as a protective mechanism to maximize cellular ATP levels and sustain cell viability as a process known as stress-induced mitochondrial hyperfusion (Gomes et al., 2011; Rambold et al., 2011). To gain insights into the functional requirement of Trak1 for mitochondrial hyperfusion during cellular stress, we treated shCTRL and shTrak1 with Hank's balanced salt solution (HBSS) to induce nutrient starvation (Fig. 2-11). Treatment of shCTRL cells with HBSS resulted in mitochondrial elongation and protection from apoptosis for up to 24 hours of nutrient starvation, while shTrak1 cells did not undergo mitochondrial elongation, remained fragmented, and underwent significant cell death after 12 hours of nutrient starvation (Fig. 2-11). A similar result was obtained with treatment under DPBS or cycloheximide (Fig. 2-12). Together these results reveal a novel function for Trak1 in normal mitochondrial fusion as well as stress-induced mitochondrial hyperfusion, which represent a pro-survival response to protect cells against certain types of stress stimuli.

2.4. Discussion

Perturbation of Trak1 function has been linked to various disease states, including hypertonia, childhood absence epilepsy, and cancer highlighting the importance of Trak1 to normal cellular

physiology. Trak1 has previously been shown to have a role in axonal mitochondrial transport and in endosome-to-lysosome trafficking. We identified a novel function for Trak1 in mitochondrial fusion and reveal a functional interaction between Trak1 and Mitofusins.

The Mitofusin proteins are essential to mitochondrial fusion. They are anchored to the outer mitochondrial membrane through a C-terminal segment and contain two N-terminal GTPase domains oriented in the cytoplasm (Fritz et al., 2001). Mitofusins form homo-oligomeric and heter-oligermic complexes on apposing mitochondrial outer membranes and use GTPase activity to drive membrane merger (Chen et al., 2003a; Meeusen et al., 2004). Cells lacking both Mfn1 and Mfn2 have no mitochondrial fusion activity and consequently their mitochondria are severely fragmented (Chen et al., 2005). Conversely, the overexpression of either Mfn1 or Mfn2 stimulates mitochondrial fusion, resulting in mitochondria with hyperfused morphology (Legros et al., 2002; Rojo et al., 2002). Although there is ample evidence that Mitofusins are required for mitochondrial fusion, the upstream pathways that bring two mitochondrial fusion partners together in close enough proximity for apposing Mitofusins to interact is not known.

Our initial experiments demonstrate that Trak1 depletion via shRNA knockdown results in mitochondrial fragmentation and reduced rates of fusion, while the overexpression of Trak1 promotes fusion. These results indicate that Trak1 directly plays a role mitochondrial fusion. One possibility is that Trak1 may functionally interact with Mitofusins to enhance or modulate its activity, since overexpression of Trak1 results in hyperfusion, while Mitofusin overexpression in Trak1 depleted cells fails to induce fusion. Furthermore, in the absence of Mitofusins the overexpression of Trak1 promotes mitochondrial clustering with apposing outer mitochondrial membranes in close contact but could not alone drive fusion. Taken together our results indicate that Trak1 acts in a pre-fusion step to mediate mitochondrial transport and tethering thus providing a mechanism to allow Mitofusin proteins on adjacent mitochondria to interact and drive membrane merger (modeled in Fig. 2-13).

Trak1 proteins are targeted to mitochondria through interaction with the integral outer mitochondrial membrane proteins, Miro1 and Miro2 (Glater et al., 2006). Miro1 and Miro2 Rho GTPases are core components of the mitochondrial trafficking machinery and enhance mitochondrial movement when overexpressed (Macaskill et al., 2009b; Saotome et al., 2008). The exogenous expression of Miro proteins can also increase mitochondrial length (Fransson et al., 2006; Saotome et al., 2008), suggesting the Miro proteins can regulate the fusion state of mitochondria. We found that when Miro proteins were overexpressed in control HeLa cells or WT MEFs mitochondrial fusion was elevated but Miro could not promote mitochondrial fusion in the absence of Trak1. One possibility is that Miro is indirectly involved in mitochondrial fusion and that the enhancement of mitochondrial fusion in Miro overexpressing cells is through the increased recruitment of Trak1 to mitochondria. Additionally, we found that overexpression of Miro proteins in Mitofusin null MEFs had no effect on mitochondrial morphology or distribution. More importantly, Miro could not mediate mitochondrial tethering, indicating that Trak1 and Miro have non-redundant roles in regulating processes of mitochondrial dynamics.

Mitochondrial fusion is cell protective and necessary to maintain mitochondrial functions (Chen et al., 2010a; Lee et al., 2004a; Tondera et al., 2009). Fusion allows the exchange of contents between healthy and dysfunctional mitochondria for replacement or repair of the damaged materials, including mtDNA. During starvation and cellular stress mitochondria elongate and hyperfuse to sustain ATP production and prevent apoptotic cell death, in a process termed stress-induced mitochondrial hyperfusion (Gomes et al., 2011; Rambold et al., 2011; Tondera et al., 2009). The genetic deletion of Mfn1 or the inner mitochondrial membrane fusion protein OPA1 inhibits stress-induced mitochondrial hyperfusion and causes cell death, thus stress-induced mitochondrial hyperfusion is a pro-survival response against cellular stress. We found that cells depleted for Trak1, which exhibit heavily fragmented mitochondria under normal growth conditions, could not undergo stress-induced mitochondrial hyperfusion under nutrient

starvation, and consequently were subject to apoptotic cell death. Cells capable of stress-induced mitochondrial hyperfusion were protected from cell death, thus Trak1 is also essential to fusion under cellular stress.

Mitochondria undergo continuous cycles of fusion and fission, which are normally balanced to maintain mitochondrial morphology and function (Cagalinec et al., 2013; Knott and Bossy-Wetzel, 2008; Twig et al., 2008). Mice lacking the fusion and fission factors: Mfn1, Mfn2, OPA1, or Drp1 die early in embryonic development (Chen et al., 2003a; Davies et al., 2007; Ishihara et al., 2009), suggesting that mitochondria fusion and fission are essential to development. In humans, point mutations in Mfn2 and OPA1 lead to the neurodegenerative diseases, Charcot-Marie-Tooth type 2A and dominant optic atrophy, respectively (Alexander et al., 2000; Delettre et al., 2000; Zuchner et al., 2004). These findings demonstrate the importance of fusion and fission regulation to cellular homeostasis and highlight their importance in health and disease. A Trak1 mutations resulting in C-terminal protein truncation causes recessively transmitted hypertonia in mice, suggesting a loss-of-function mechanism in disease etiology. However, as of yet no mechanistic explanation exists for how the Trak1 hyrt mutation affects cellular process. We found that Trak1 hyrt mutations affects targeting to mitochondria, resulting in partial mislocalization away from mitochondria to the cytosol, and impaired the ability of Trak1 hyrt to promote mitochondrial fusion. Our results suggest a novel mechanism to hypertonia pathogenesis and may provide insights into new therapeutics.

In conclusion, our findings reveal a novel function for Trak1 in mitochondrial fusion and provide new insights in to the regulation of mitochondrial dynamics.

2.5. Materials and methods

2.5.1. Cell culture, Transfection, and Immunoblotting analysis

Cells were grown in Dulbecco's modified Eagle medium (DMEM; GIBCO) with 10% (v/v) fetal bovine serum (Atlanta Biologicals) and 1% (v/v) penicillian streptomycin (Fisher) in a humidified incubator at 37° C with 5% CO2. HeLa cells were transfected with the indicated plasmids using Lipofectamine 2000 reagent (Invitrogen), while MEF cells were transfected using Fugene HD (Promega), both in accordance with the manufacturer's instructions. For generation of stable Trak1 knockdown cell lines, Trak1 shRNA plasmids were transfected in HeLa cells followed by selection with 2.5 μg/ml puromycin (Research Products International). Single puromycin-resistant colonies were isolated for culture and subsequently assayed for expression of endogenous Trak1 by Western blotting. For western blot analysis cells were homogenized in 1% SDS and then subjected to SDS-PAGE. Proteins were transferred onto nitrocellulose membranes and then probed with the indicated primary antibodies and horseradish peroxidase-conjugated second antibodies. Antibody binding was detected using the enhanced chemiluminescence (ECL) system.

2.5.2. Plasmids and Antibodies

Conventional molecular biological techniques were used to generate the GFP-tagged Trak1 expression constructs: Trak1 WT (residues 1–953), and Trak1 hyrt (residues 1–824) as previously described (Webber et al., 2008). The rescue constructs encoding shRNA resistant Trak1 WT and Trak1 hyrt were generated by site-directed mutagenesis to make two or three silent third-codon substitutions within the shRNA-targeted region of the Trak1 transcript without altering the Trak1 amino acid sequence. ShRNA constructs against human Trak1 (NM_014965.2-876s1c1 and NM_014965.2-1392s1c1) and a non-targeting control construct (SHC001) were from Sigma. The full-length Miro1 and Miro2 constructs were a gift from Dr. Pontus Aspenstrom (Ludwig

Institute for Cancer Research, Uppsala University, Sweden). The full-length Mfn1 and Mfn2 constructs were a gift from Dr. David Chan (California Institute of Technology). The pDsRed2-Mito construct is available commercially (Clontech) and the mito-Dendra2 construct was a gift from Dr. Michael T. Ryan (La Trobe University, Australia). Rabbit polyclonal anti-Trak1 antibody was generated against the synthetic peptide ILTSGILMGAKLPKQTSLR, corresponding to residues 935–953 of humanTrak1 and was affinity-purified as previously described (Webber et al., 2008). Other primary antibodies used in this study include: anti-TOM20 (Santa Cruz); anti-GFP (B2, Santa Cruz); anti-Myc (9E10); anti-HSP60 (Stressgen); anti-GAPDH (Cell Signaling); and anti-Actin (C4, Sigma). Horseradish-peroxidase-conjugated and FITC, or TRITC-conjugated secondary antibodies were from Jackson Immunoresearch Laboratories, Inc.

2.5.3. Immunofluorescence confocal microscopy

For immunofluorescence confocal microscopy, cells were grown on poly-L-Lysine coated coverslips and fixed in 4% paraformaldehyde for 20 minutes. Cells were permeabilized with 0.1% saponin, blocked with 4% horse serum in PBS, and stained with the indicated primary antibodies and secondary antibodies conjugated to FITC or TRITC. For mitochondrial staining MitoTracker Deep Red FM (Life Technologies) was added to living cells at a final concentration of 25 nM for 15 minutes and washed once with pre-warmed media for 30 minutes at 37° C before fixation. Nuclei were visualized with DAPI from the Prolong Gold antifade mountant with DAPI (Life Technologies). Image acquisition was conducted at room temperature using a Nikon Eclipse Ti confocal laser-scanning microscope as previously described (Lee et al., 2012). Images were exported in TIFF format with the Nikon EZ-C1 viewer and processed using Adobe Photoshop CS5 (Adobe Systems, Inc.) to adjust brightness and to produce the figures.

2.5.4. MitoDendra2 assay and Analysis of fusion

HeLa cells were seeded in glass-bottom MatTek culture dishes and transfected with Mito-Dendra2. Cells were subjected to live-cell time-lapse imaging 24 hours after transfection in a humidified chamber with 5% CO_2 maintained at 37° C on a heated stage. In a region of interest (ROI) a subset of Mito-Dendra2 was irreversibly converted from green (excitation at 488 nm and emission at 515 nm) to red (excitation at 561 nm and emission at 590 nm) fluorescent states by irradiation with a 408 nm laser at 2% intensity for 10-15 iterations. Following photoconversion live cell time-lapse images were captured every 30 seconds for at least 20 minutes in a single optical z-section with the 488 laser at 0.1% intensity and the 561 laser at 0.5% intensity to prevent spontaneous photoactivation and photobleaching.

In a similar and consistent ROI, a subset of mitochondria was photoconverted in shCTRL and shTrak1 stable cells. Quantification of colocalization was achieved using Image J software (http://rsb.info.nih.gov/ij/index.html) to determine the percentage of the area of red fluorescent mitochondria overlapping with green fluorescent mitochondria to approximate the rate of fusion over time as previously described (Magrane et al., 2012).

2.5.5. 3D-Structured Illumination (SIM) Microscopy

For 3D-SIM analyses, cells were fixed and stained as described for immunofluorescence confocal microscopy. Images were acquired as previous described (Lee et al., 2012) using the Nikon N-SIM microscopy system on an Eclipse Ti inverted microscope (Nikon Instruments Inc., Melville, NY). SIM data was reconstituted using the following parameters: Structured illumination contrast = 2.0; Apodization Filter = 1.0; Width of 3D-SIM filter = 0.20. Image acquisition, analysis, and rendering were achieved using the Nikon Elements software and each image represents a single Z section of a 3D SIM image.

2.5.6. Electron Microscopy

For ultrastructure studies, monolayer cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) followed by post-fixation with 1% osmium and 1.5% potassium ferrocyanide in the same buffer. Cells were then dehydrated in ethanol and embedded in Eponate 12 resin. Ultrathin sections were cut at 70 nm with a ultramicrotome, stained with 5% uranyl acetate and 2% lead citrate, and then examined with a Hitachi H-7500 transmission electron microscope equipped with a SIA L12C 16 megapixel CCD camera.

2.5.7. Subcellular fractionation

HeLa cells expressing the indicated plasmids were subjected to subcellular fractionation to separate mitochondria containing and cytosol fractions as described previously (Giles et al., 2009; Lee et al., 2011). Briefly, cells were homogenized in 1ml homogenization buffer (250 mM sucrose, 20 mM HEPES, pH 7.4, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, and 1 mM EGTA) containing protease inhibitors using dounce homogenization and subsequently centrifuged at 1,000 g to pellet nuclei and unbroken cells. The postnuclear supernatant was then subjected to centrifugation at 10,000 g for 15 minutes to isolate cytosol (supernatant) and mitochondria containing (pellet) fractions. Equal percentage of lysates from each fraction: Total, Cytosolic, and Mitochondrial were subjected western blot. For quantification of subcellular fractionation the relative levels of Trak1 were determined by measuring the intensity of the Trak1 band from the western blot image using the NIH Image J software. The level of Trak1 in each fraction relative to the total level in the post-nuclear supernatant was quantified from at least three independent experiments.

2.5.8. Stress-Induced Mitochondrial Hyperfusion and Apoptosis Detection

To induce stress-induced mitochondrial hyperfusion cells were treated with 10 μM cycloheximide (CHX, Sigma-Aldrich), Dulbecco's PBS (DPSB, Life Technologies cat. # 14287-080), or Hank's balanced salt solution (HBSS, Life Technologies cat. # 24020-117) for 0, 6, 12 and 24 hours as previously described (Rambold et al., 2011; Tondera et al., 2009). At the indicated time points, cells were fixed and immunostained for TOM20, stained for DAPI to visualize nuclei, and subjected to fluorescence confocal microscopy. Positive stress-induced mitochondrial hyperfusion was quantified based on presence of highly interconnected tubular mitochondria. For apoptosis, morphological assessment was performed on DAPI stained nuclei as described previously (Chen et al., 2010b). The percentages of cells with nuclear fragmentation and chromatin condensation were scored as apoptotic.

2.5.9. Analyses of mitochondrial morphology

For analysis of mitochondrial morphology cells were categorized according to these criteria: Normal: a mixed population of interconnected tubular mitochondria and non-connected shorter mitochondria; Fragmented: the majority of mitochondria are not connected and are spherical rather that tubular in appearance; Hyperfused/Elongated: mitochondria are highly interconnected, with very few to no non-connected mitochondria. Mitochondria were categorized as enlarged if greater than 2 μM in width.

2.5.10. Statistical Analyses

Data were analyzed by a Student's t-test or a one-way analysis of variance (ANOVA) with a Tukey's *post hoc* test using the Sigma-Plot software (Systat Software, Inc.). Results are expressed

as mean ± SEM obtained from at least 3 independent experiments. A p-value of < 0.05 was considered statistically significant.

2.6. Acknowledgements

This work was supported by the National Institutes of Health (GM103613 to L.L. and AG034126 to L.S.C.) and the Emory University Research Committee (SK46673 to L.L.). C.A.L. was a trainee supported by NIH Training Grant T32 GM008367. We thank Emory University Integrated Cellular Imaging Microscopy Core (supported in part by NIH grant NS055077) and Emory Robert P. Apkarian Integrated Electron Microscopy Core for providing instrumentation and support.

Conflict of Interest statement. None declared

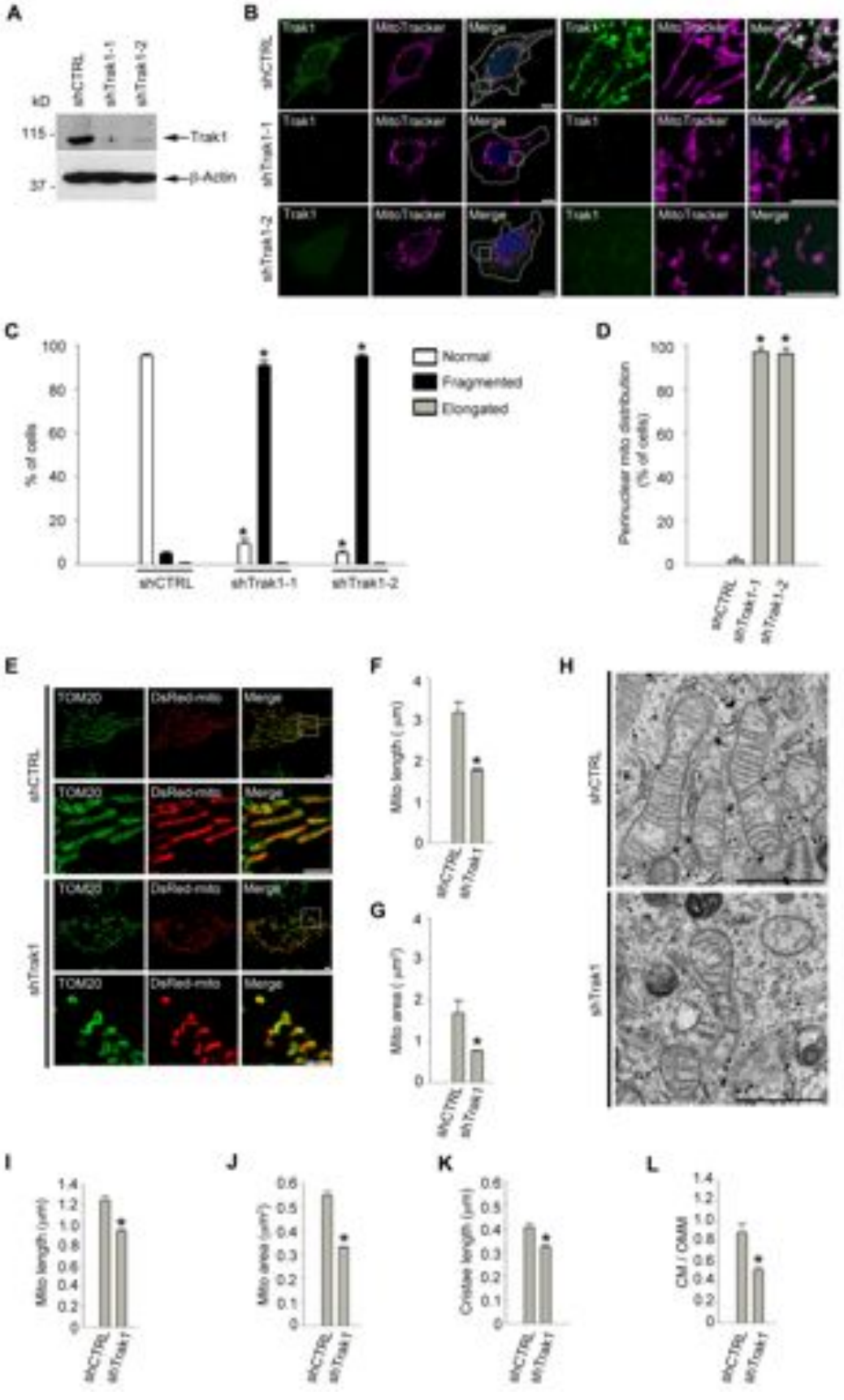

A
shCTRL
shTrak1-1
shTrak1-2
kD
115 -
37 -
Trak1
β-Actin
B
shCTRL
shTrak1-1
shTrak1-2
Trak1
MitoTracker
Merge
C
% of cells
Normal
Fragmented
Elongated
shCTRL
shTrak1-1
shTrak1-2
D
Perinuclear mito distribution (% of cells)
E
shCTRL
shTrak1
TOM20
DsRed-mito
Merge
F
Mito length (μm)
G
Mito area (μm²)
H
shCTRL
shTrak1
I
Mito length (μm)
J
Mito area (μm²)
K
Cristae length (μm)
L
CM / OMM

Figure 2-1. **Trak1 depletion results in mitochondrial fragmentation and loss of mitochondria at the cell periphery.** (A) Two independent clonal Trak1 shRNA lines (shTrak1-1 and shTrak1-2) and a non-targeting shRNA cell line (shCTRL) were generated in HeLa cells. Western blotting of whole-cell lysates with anti-Trak1 antibody was used to assess relative levels of Trak1 protein. Anti-β-Actin immunoblotting was used as a loading control. (B) ShTrak1-1, shTrak1-2, and shCTRL cell lines were stained with MitoTracker Deep Red (purple) to visualize mitochondria and immunostained with anti-Trak1 antibody (green). Nuclei were visualized by DAPI stain (blue) in merged images. Insets show an enlarged view of boxed area. Scale bar = 10 μm, 5 μm for inset. (C) Scoring of mitochondrial network morphologies based on criteria of normal, fragmented, or elongated from shCTRL and shTrak1 cell lines. (D) Quantification of percent of cells with perinuclear mitochondrial distribution for the indicated conditions. Data represents mean ± SEM (n = 30-40 cells per group per experiment) from three independent experiments. *, $P < 0.05$ versus shCTRL, one-way analysis of variance with a Tukey's post hoc test. (E) ShCTRL and shTrak1 stable HeLa cells transfected with DsRed-mito (red) and immunostained for endogenous mitochondrial protein, TOM20 (green) were visualized by SIM microscopy. Insets show an enlarged view of boxed area. Quantification of mitochondrial length (F) and area (G) from SIM images. Data represents mean ± SEM (n = 3 cells per group per experiment) from three independent experiments. *, $P < 0.05$ versus shCTRL, t-test. Scale bar = 2 μm. (H) Mitochondria from shCTRL and shTrak1 stable HeLa cells were visualized by transmission electron microscopy. Quantification of mitochondrial length (I) and area (J) from TEM images. Quantification of cristae length (K) and cristae membrane to mitochondrial outer membrane ratio (L) from TEM images. Data represent mean ± SEM (n = 84 mitochondria for shCTRL, n = 173 mitochondria for shTrak1) from at least five cells. *, $P < 0.05$ versus shCTRL, t-test. Scale bar = 1 μm.

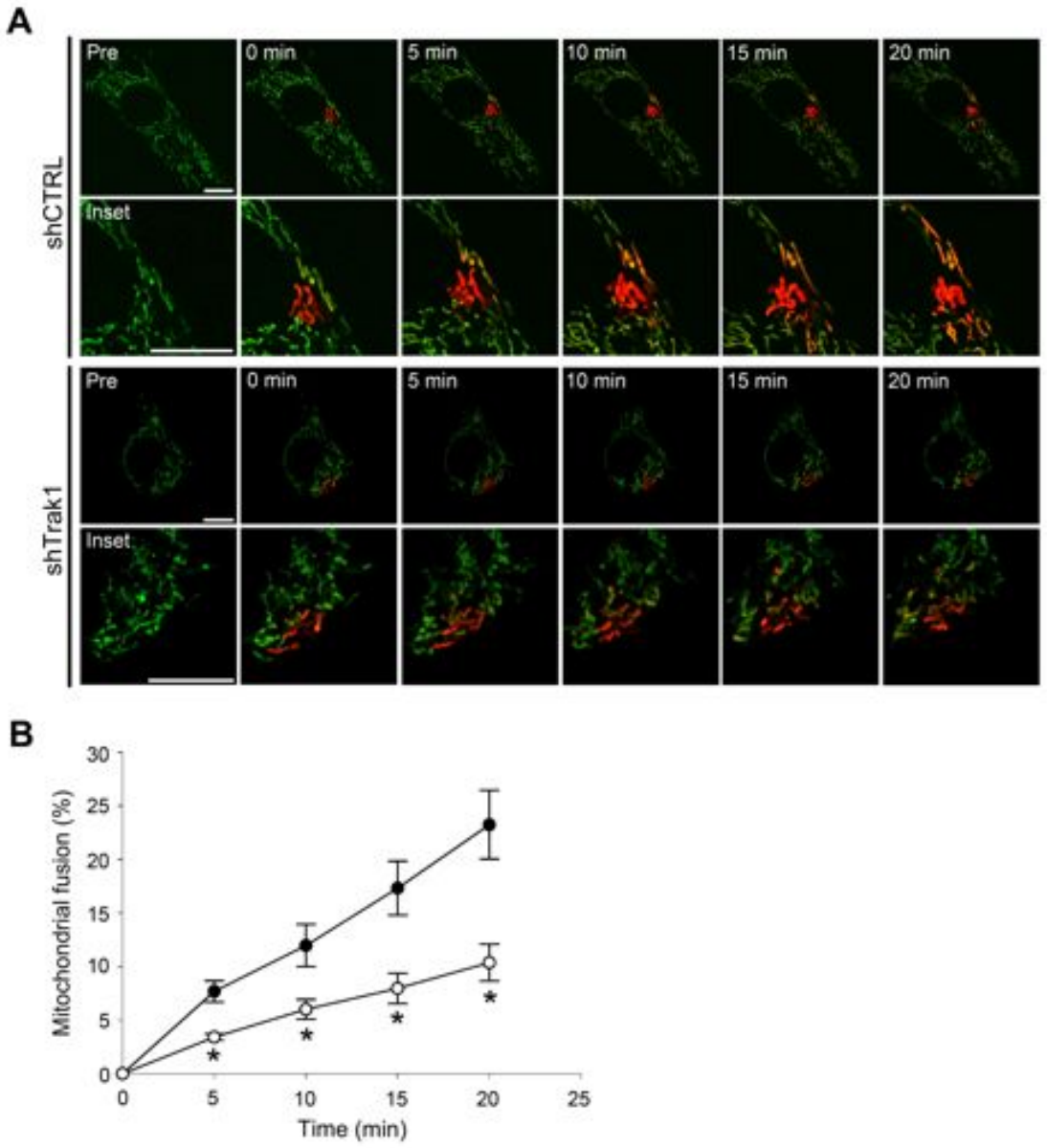


Figure 2-2. **Trak1 depletion impairs mitochondrial fusion.** (A) Live cell time-lapse confocal imaging was used to directly assess fusion and fission events in shTrak1 and shCTRL stable HeLa cells transfected with photoswitchable mito-Dendra fluorescent protein. A subset of mitochondria within a region of interest (ROI) was irreversibly photoconverted from green to red at time 0 and imaged for 20 minutes. Yellow fluorescence indicates the mixing of red and green mitochondria through fusion. Scale bar = 10 μm. (B) Mitochondrial fusion was assessed by quantification of colocalization to determine the percentage of the area of red fluorescent

mitochondria overlapping with green fluorescent mitochondria at the indicated time points. Data represents mean ± SEM (n = 4 cells per group per experiment) from three independent experiments. *, $P < 0.05$ versus shCTRL, t-test.

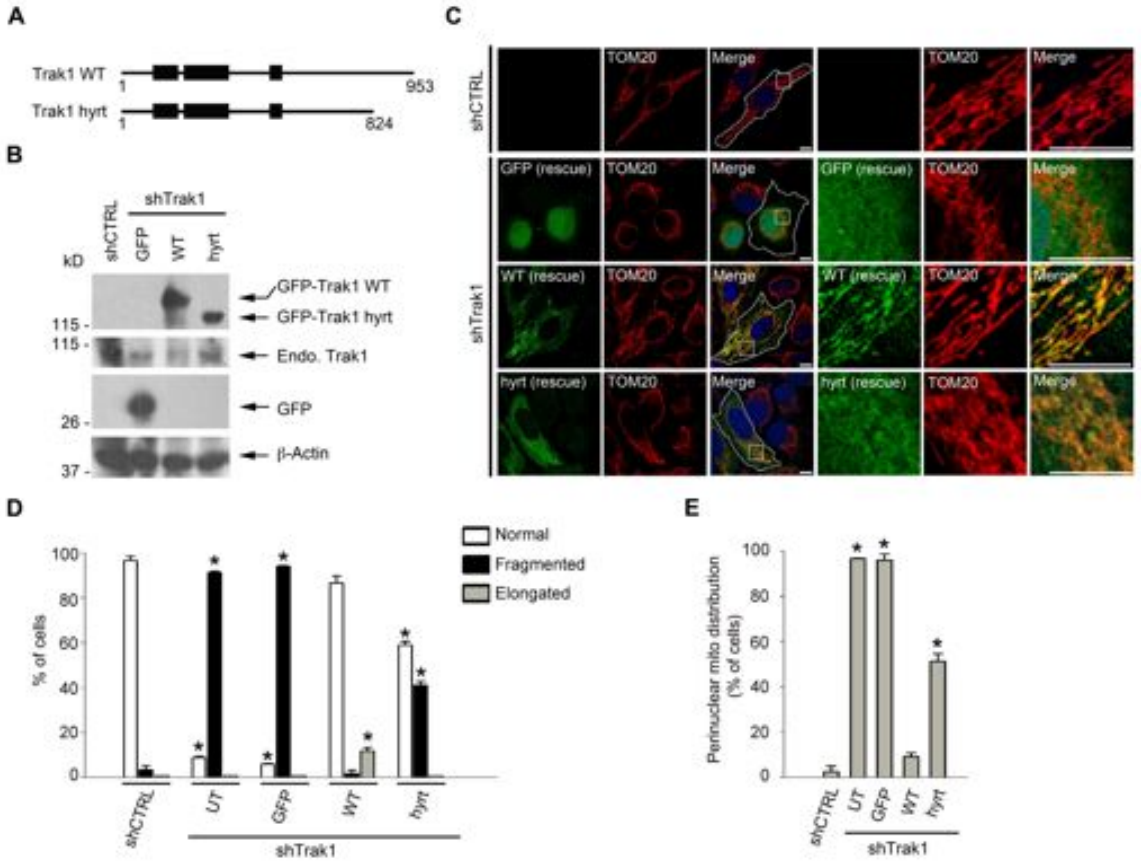


Figure 2-3. **Trak1 WT but not Trak1 hyrt fully rescues fragmentation and perinuclear localization of mitochondria in shTrak1 cells.** (A) Schematic representation of the domain structures of Trak1 WT and Trak1 hyrt. (B) Expression levels of GFP-tagged shRNA resistant rescue constructs were determined by western blotting with anti-GFP antibody. Anti-Trak1 immunoblotting was used to show shTrak1 cells are depleted for endogenous Trak1 expression compared to shCTRL cells. Anti-β-Actin was used as a loading control. (C) ShCTRL cells were stained for endogenous TOM20 to visualize mitochondria. ShTrak1 cells were transfected with either GFP control or the indicated GFP-tagged Trak1 rescue constructs (green) and mitochondria were visualized by TOM20 immunostaining (red). Nuclei were visualized by DAPI stain (blue) in merged images. Insets show an enlarged view of boxed area. (D) Scoring of mitochondrial network morphologies based on criteria of normal, fragmented, or elongated for the indicated conditions. (E) Quantification of percent of cells with perinuclear mitochondrial distribution for

the indicated conditions. Data represents mean ± SEM (*n* = 31-40 cells per group per experiment) from three independent experiments. *, $P < 0.05$ versus GFP; #, $P < 0.05$ versus Trak1 WT, one-way analysis of variance with a Tukey's post hoc test. Scale bar = 10 μm. UT = untransfected.

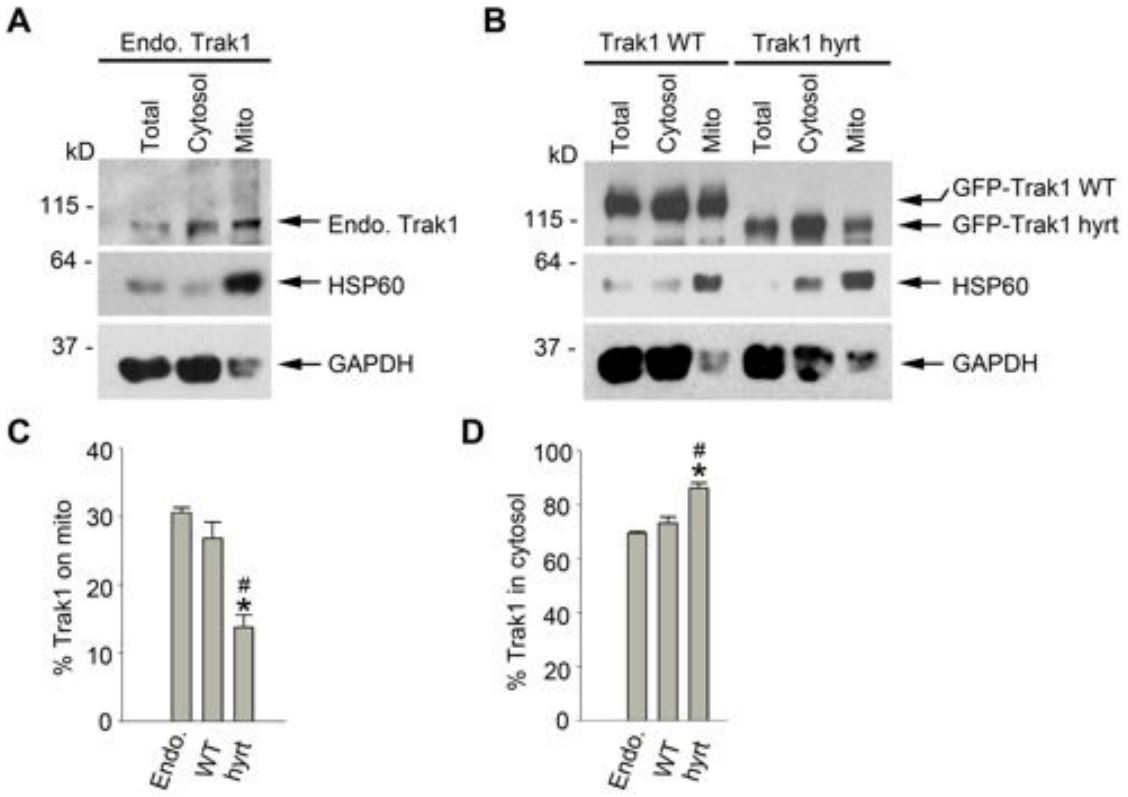


Figure 2-4. **Trak1 hyrt mutant is mislocalized from mitochondria to cytosol.** Postnuclear supernatant (Total) from either untransfected cells (A) or cells transfected with the indicated GFP-tagged Trak1 constructs (B) were separated into cytosol and mitochondria containing fractions. Equal percentages of each fraction were subjected to immunoblot analysis. Anti-HSP60 was used to indicate the mitochondrial fraction while anti-GAPDH was used to indicate the cytosolic fraction. The percentage of Trak1 in the mitochondrial fraction (C) and the cytosolic fraction (D) was quantified relative to the amount in the corresponding postnuclear supernatant fraction (Total) from three independent experiments and shown as mean ± SEM from three independent experiments. *, $P < 0.05$ versus endogenous Trak1; #, $P < 0.05$ versus Trak1 WT, one-way analysis of variance with a Tukey's post hoc test.

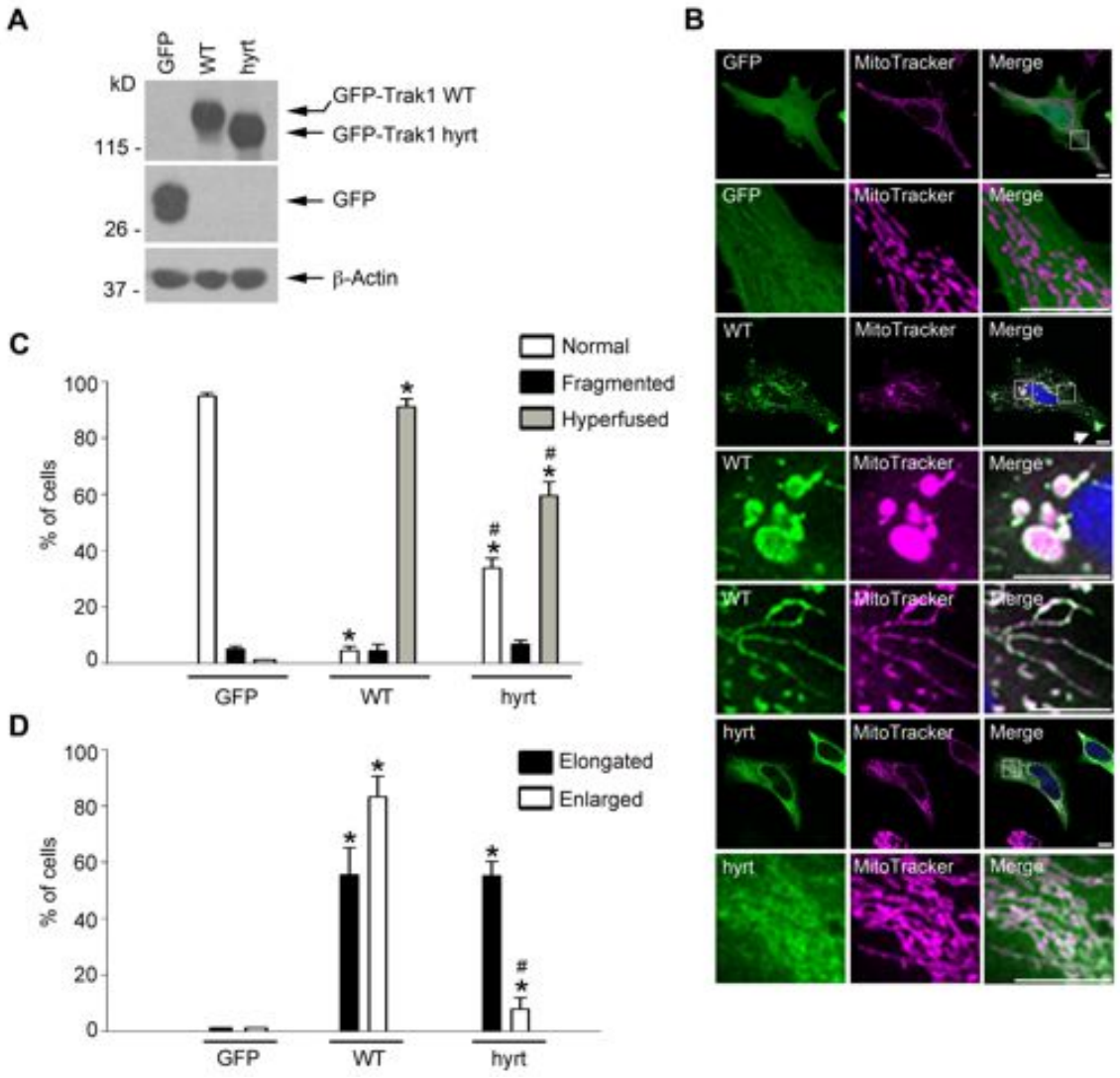


Figure 2-5. **Trak1 WT overexpression promotes mitochondrial hyperfusion while Trak1 hyrt is less effective at inducing hyperfusion.** (A) Expression levels of GFP-tagged proteins were assessed by western blotting with anti-GFP antibody. β-Actin immunoblotting were used as loading controls. (B) HeLa cells expressing the indicated GFP-tagged proteins (green) were stained with MitoTracker Deep Red (purple) to visualize mitochondria. Nuclei were visualized by DAPI (blue) stain. Insets show an enlarged view of boxed area. Arrowhead indicates mitochondria concentrated at the cell periphery in a Trak1 WT overexpressing cell. Scale bar =

10 μm. (C) Scoring of mitochondrial network morphologies based on criteria of normal, fragmented, or hyperfused for the indicated conditions. (D) Scoring of hyperfused mitochondria as either elongated (highly connected) or enlarged (greater than 2 uM in width). Data represents mean ± SEM (*n* = 40-50 cells per group per experiment) from three independent experiments. *, $P < 0.05$ versus GFP; #, $P < 0.05$ versus Trak1 WT (WT), one-way analysis of variance with a Tukey's post hoc test.

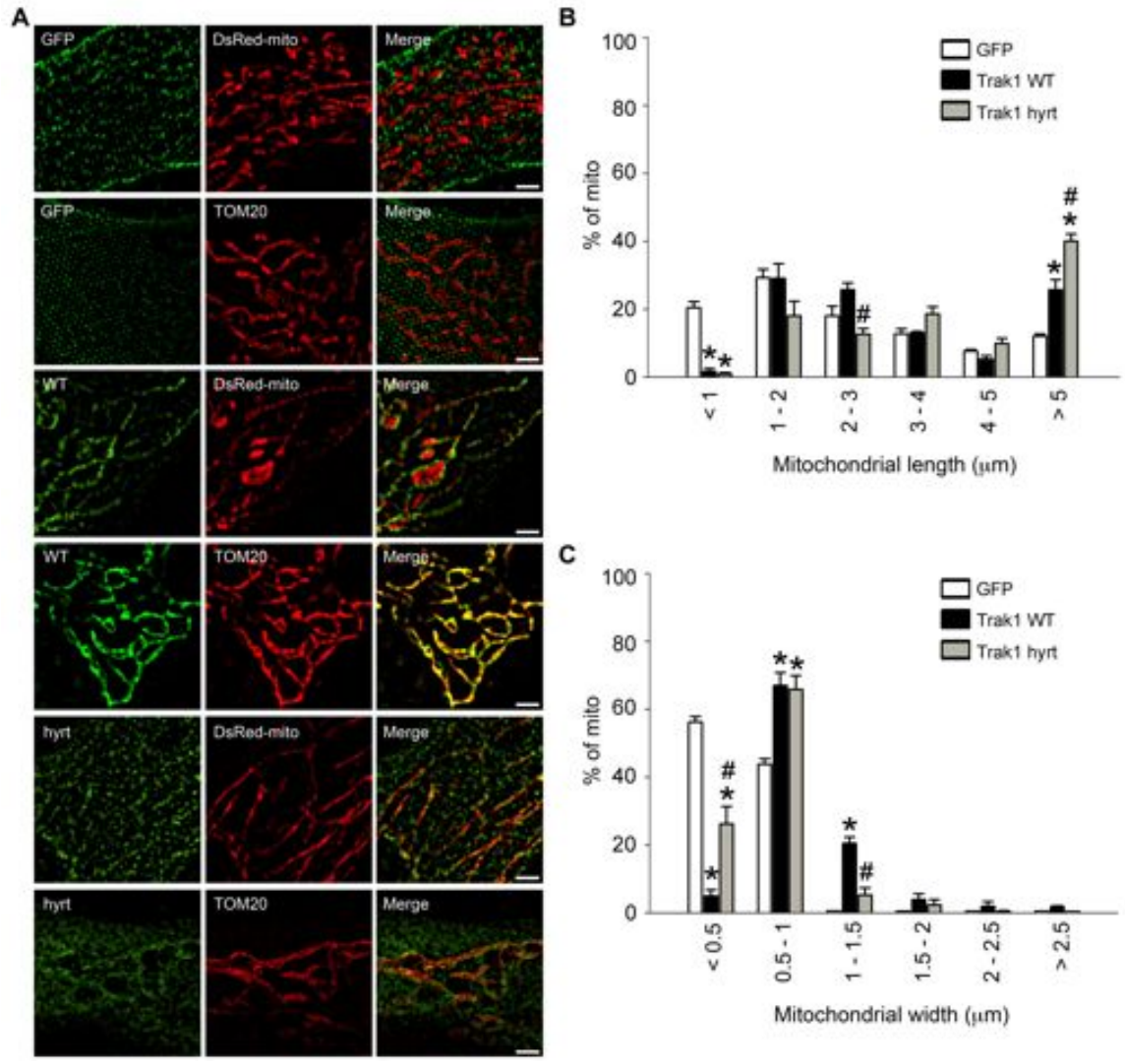


Figure 2-6. **High resolution SIM microscopy indicates Trak1 overexpression increases mitochondrial size.** Mitochondria from HeLa cells expressing GFP or the indicated GFP-tagged Trak1 proteins (green) were labeled with DsRed-mito (red) or endogenous TOM20 immunostaining (red) and subsequently analyzed by 3D-SIM. Scale bar = 2 μm. Quantification of the percent of mitochondria with different ranges of length (B) and width (C) from SIM images. Data represents mean ± SEM (n = 3 cells per group per experiment) from three independent experiments. *, $P < 0.05$ versus GFP Vector; #, $P < 0.05$ versus Trak1 WT, one-way analysis of variance with a Tukey's post hoc test.

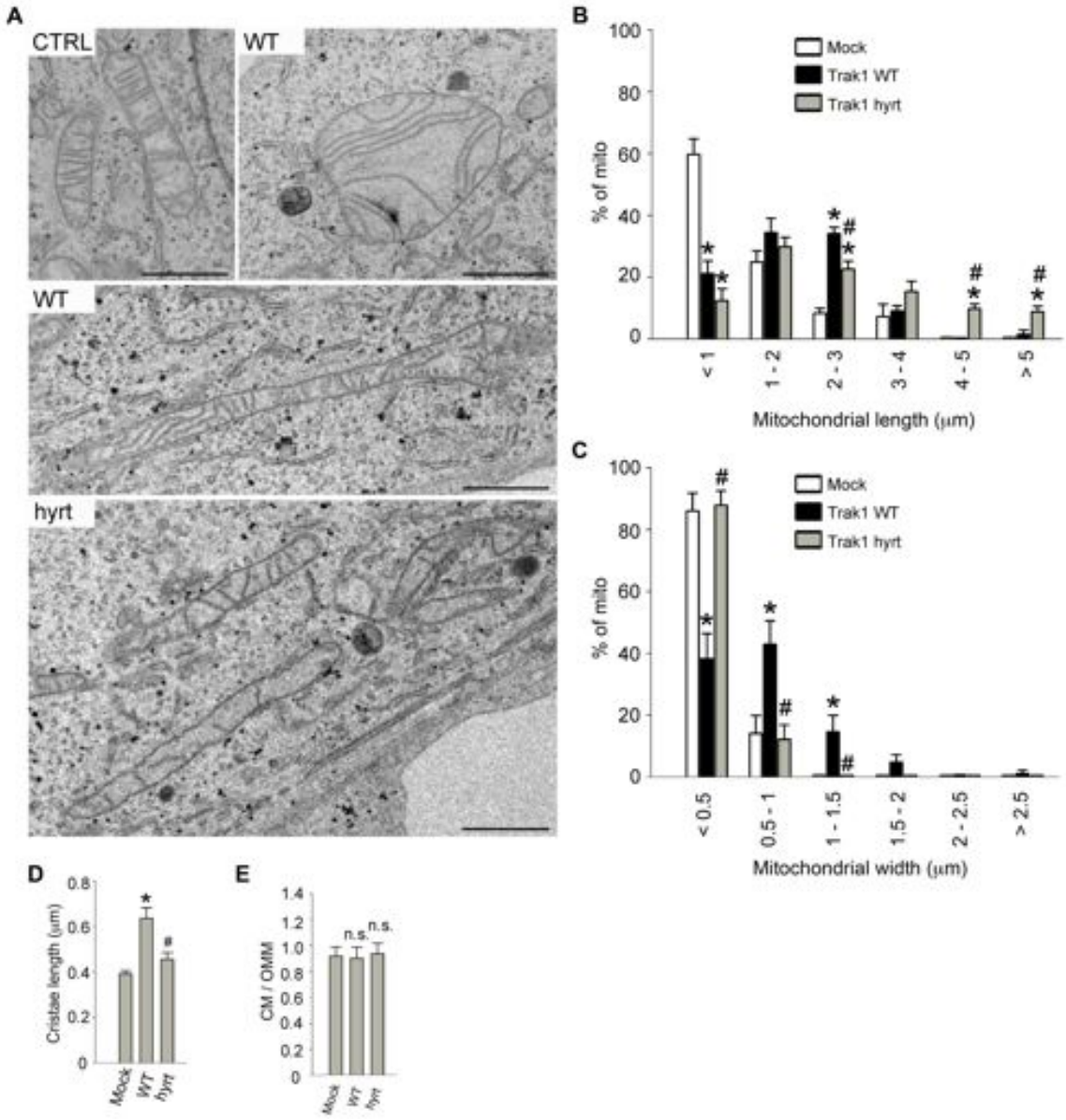
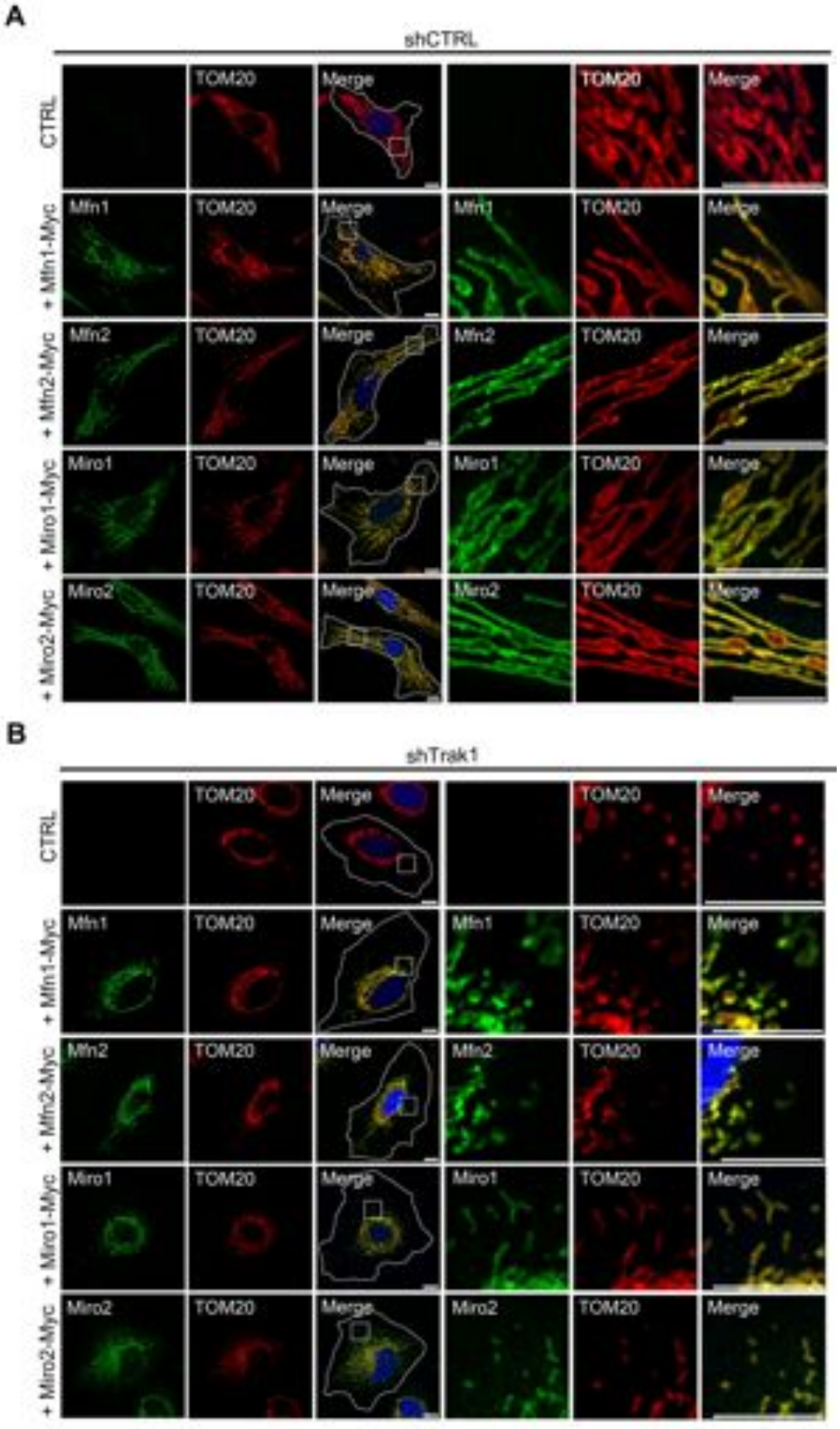


Figure 2-7. **Ultra structural analysis by TEM indicates Trak1 overexpression causes increased mitochondrial area and disrupted cristae organization.** (A) HeLa cells were either mock transfected (CTRL) or transfected with plasmid encoding GFP-Trak1 WT (WT), or GFP-Trak1 hyrt (hyrt) and prepared for TEM at 24 hours post-transfection. Scale bar = 1 μm. Quantification of the percent of mitochondria with different ranges of length (B) and width (C) from TEM images. Quantification of cristae length (D), and cristae membrane to mitochondrial

outer membrane ratio (E) from TEM images. Data represent mean ± SEM (n = 112 mitochondria for Mock, n = 108 mitochondria for Trak1 WT, and n = 123 mitochondria for Trak1 hyrt) from at least seven cells. *, $P < 0.05$ versus Mock; #, $P < 0.05$ versus Trak1 WT; n.s., not significant, one-way analysis of variance with a Tukey's post hoc test.


A
shCTRL
CTRL
+ Mfn1-Myc
+ Mfn2-Myc
+ Miro1-Myc
+ Miro2-Myc
TOM20
Merge
Mfn1
Mfn2
Miro1
Miro2
B
shTrak1

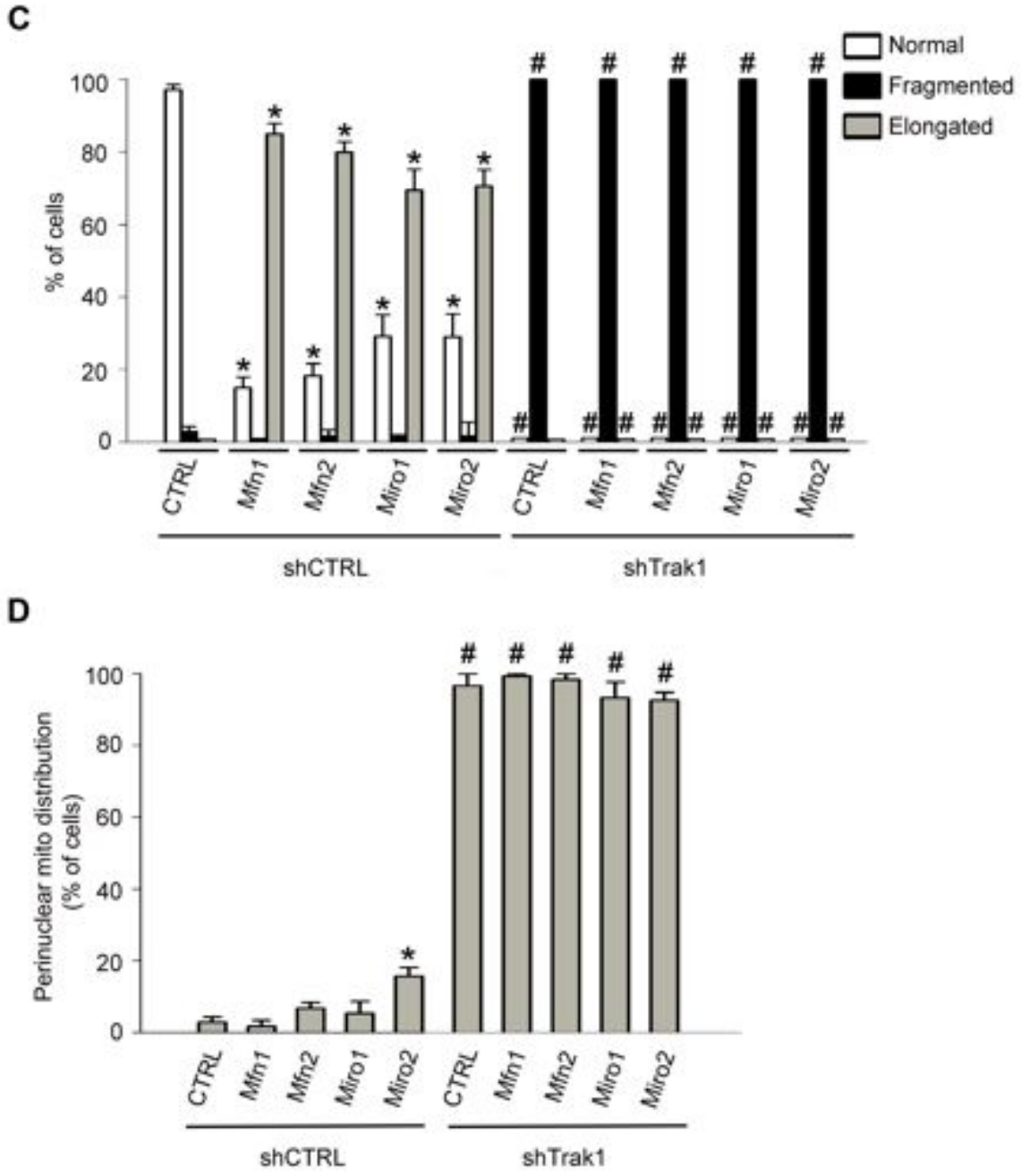
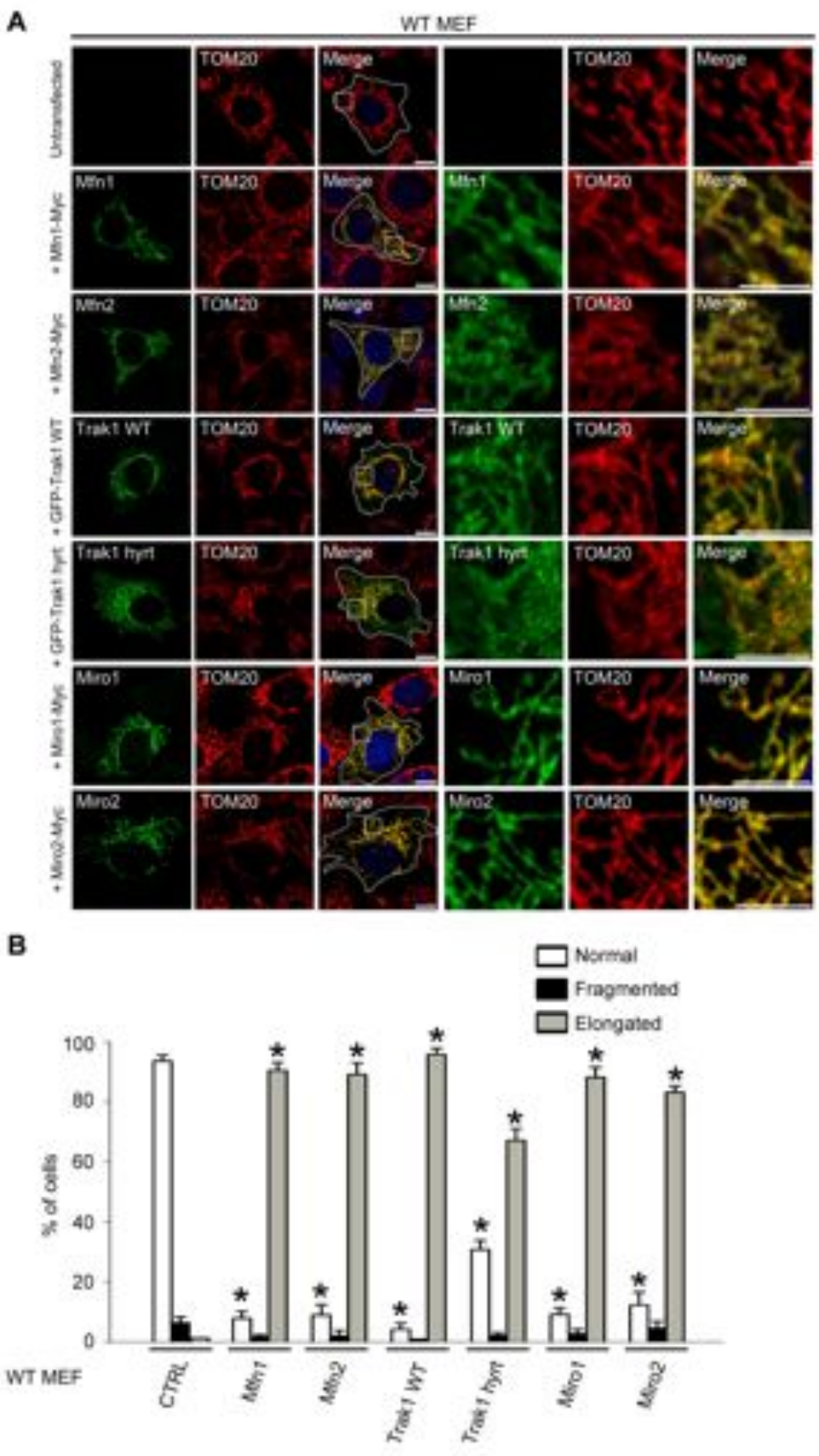


Figure 2-8. **Mitochondrial fragmentation in Trak1 depleted cells cannot be rescued by Mitofusin or Miro overexpression.** ShCTRL (A) or shTrak1 (B) stable HeLa cells were transfected with the indicated Myc-tagged Mitofusin or Miro constructs and then double immunostained with anti-Myc antibody (green) and anti-TOM20 (red) to visualize mitochondria. Nuclei were visualized by DAPI (blue) stain. Insets show an enlarged view of boxed area. Scale

bar = 10 μm. (C) Scoring of mitochondrial network morphologies based on criteria of normal, fragmented, or elongated for the indicated conditions. (D) Quantification of percent of cells with perinuclear mitochondrial distribution. Data represents mean ± SEM (*n* = 35-45 cells per group per experiment) from three independent experiments. *, $P < 0.05$ versus the corresponding CTRL, #, $P < 0.05$ versus the corresponding shCTRL condition one-way analysis of variance with a Tukey's post hoc test.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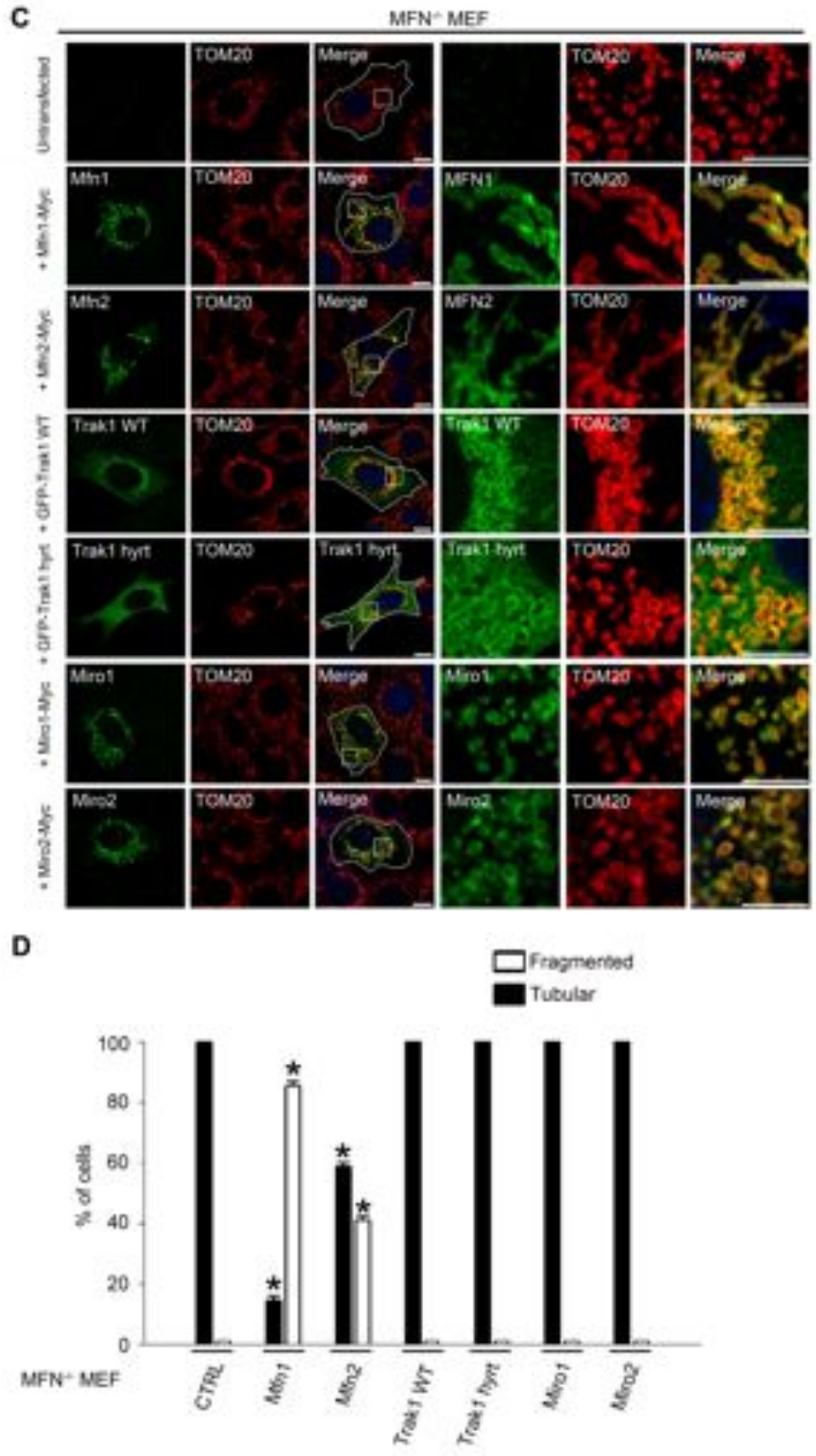
WT MEF
Untransfected
+ Mfn1-Myc
+ Mfn2-Myc
+ GFP-Trak1 WT
+ GFP-Trak1 hyrt
+ Miro1-Myc
+ Miro2-Myc
TOM20
Merge
Mfn1
Mfn2
Trak1 WT
Trak1 hyrt
Miro1
Miro2
B
Normal
Fragmented
Elongated
% of cells
100
80
60
40
20
0
WT MEF
CTRL
Mfn1
Mfn2
Trak1 WT
Trak1 hyrt
Miro1
Miro2


C
MFN⁻/⁻ MEF
Untransfected
+ Mfn1-Myc
+ Mfn2-Myc
+ GFP-Trak1 WT
+ GFP-Trak1 hyrt
+ Miro1-Myc
+ Miro2-Myc
TOM20
Merge
Mfn1
Mfn2
MFN1
MFN2
Trak1 WT
Trak1 hyrt
Miro1
Miro2
D
Fragmented
Tubular
% of cells
100
80
60
40
20
0
MFN⁻/⁻ MEF
CTRL
Mfn1
Mfn2
Trak1 WT
Trak1 hyrt
Miro1
Miro2

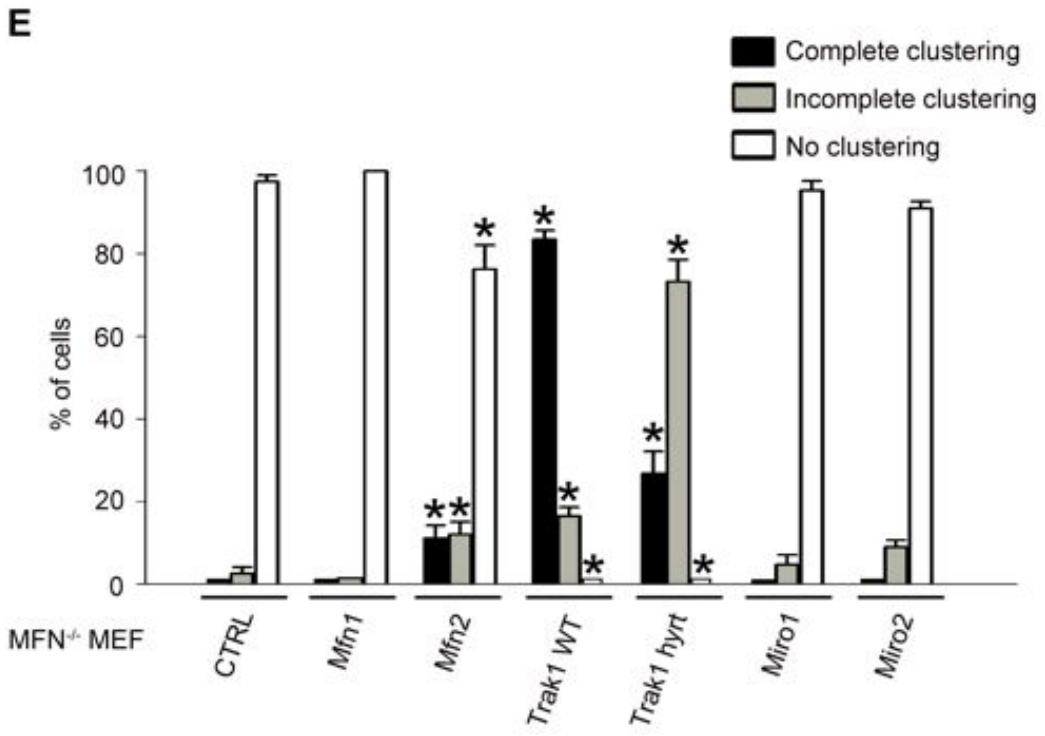


Figure 2-9. **Trak1 overexpression causes mitochondrial clustering in Mitofusin null MEFs.** WT (A) and $MFN^{-/-}$ MEFs (C) were either untransfected or transfected with the indicated plasmids. Myc-tagged proteins were immunostained with anti-Myc antibody (green). Immunostaining with anti-TOM20 (red) was used to visualize mitochondria. Nuclei were visualized by DAPI (blue) stain. Insets show an enlarged view of boxed area. Scale bar = 10 μm, 5 μm for insets. (B) Scoring of mitochondrial network morphologies in WT MEFs based on criteria of normal, fragmented, or elongated for the indicated conditions. (D) Scoring of mitochondrial morphologies in $MFN^{-/-}$ MEFs based on criteria of fragmented or tubular. (E) Scoring of mitochondrial distributions in $MFN^{-/-}$ MEFs based on criteria of complete clustering, incomplete clustering, or no clustering. Data represents mean ± SEM (n = 35-45 cells per group per experiment) from three independent experiments. *, $P < 0.05$ versus CTRL, one-way analysis of variance with a Tukey's post hoc test.

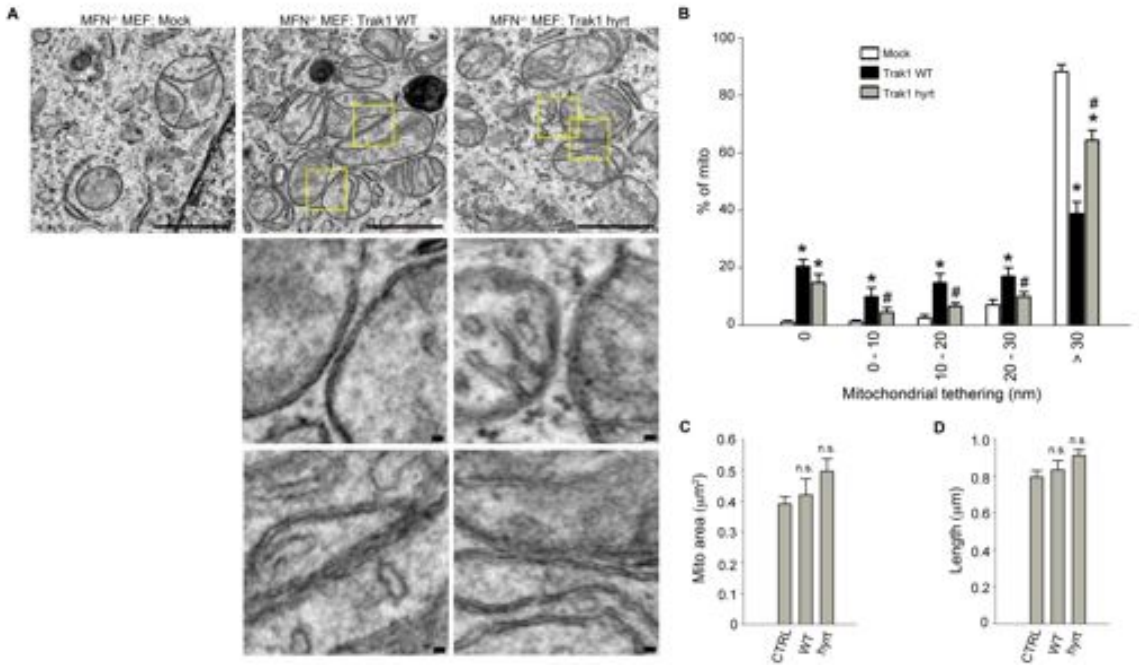


Figure 2-10. **Trak1 promotes mitochondrial tethering.** (A) MFN$^{-/-}$ MEFs were transfected with the indicated Trak1 constructs and analyzed by TEM. (B) Quantification of the percent of mitochondria with different ranges of mitochondria-mitochondria contacts (mitochondrial tethering). Quantification of mitochondrial area (C) and length (D) from TEM images. Scale bar = 1 μm, 30 nm for insets. Data represent mean ± SEM (n = 320 mitochondria for Mock, n = 258 mitochondria for Trak1 WT, and n = 269 mitochondria for Trak1 hyrt) from at least eleven cells. *, $P < 0.05$ versus Mock; #, $P < 0.05$ versus Trak1 WT; n.s., not significant, one-way analysis of variance with a Tukey's post hoc test.

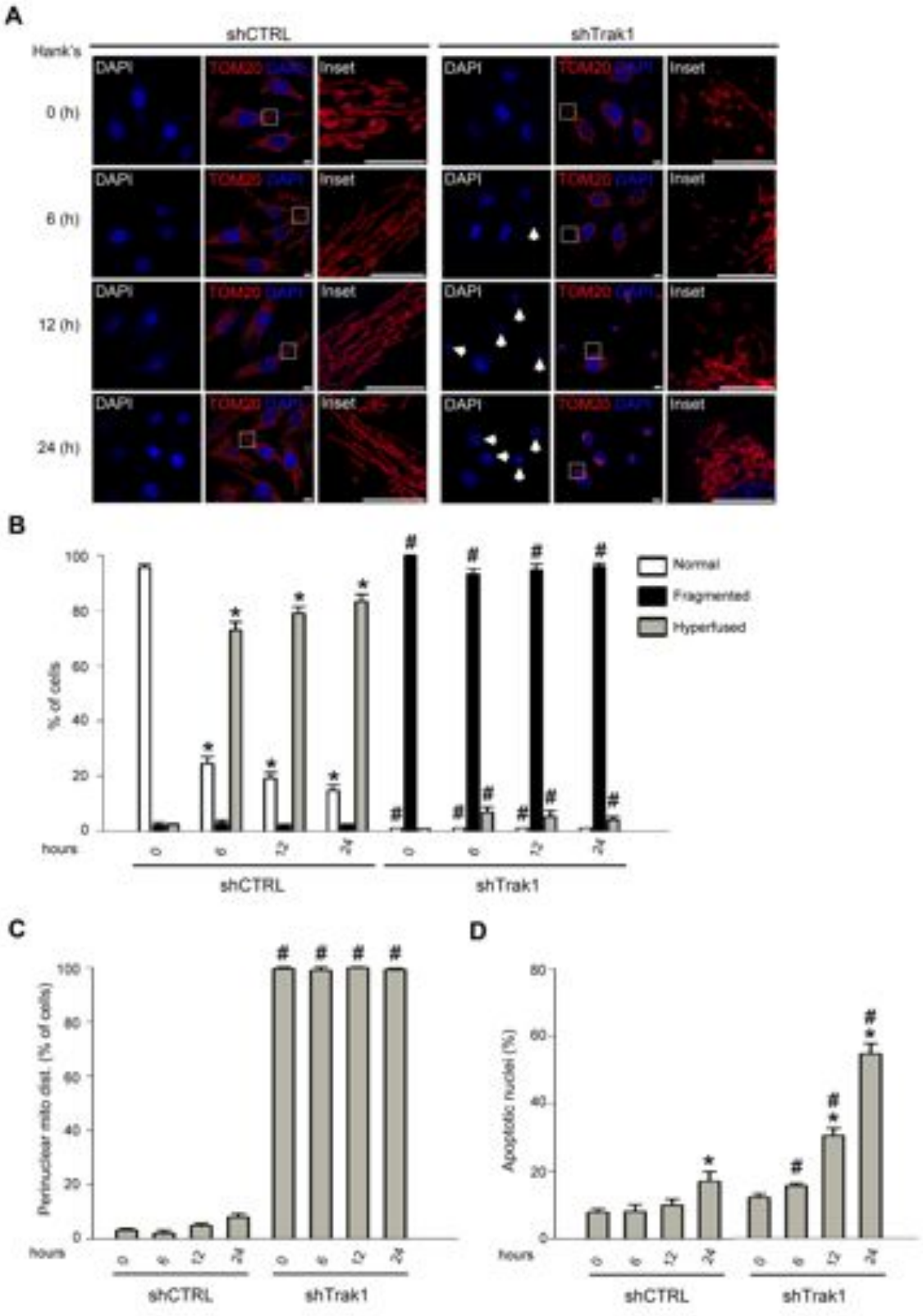

A
shCTRL
shTrak1
Hank's
0 (h)
6 (h)
12 (h)
24 (h)
DAPI
TOM20
Inset
B
Normal
Fragmented
Hyperfused
% of cells
hours
shCTRL
shTrak1
C
Perinuclear mito dist. (% of cells)
D
Apoptotic nuclei (%)

Figure 2-11. **Nutrient deprivation-induced mitochondrial hyperfusion requires Trak1.** (A) ShCTRL and shTrak1 stable HeLa cells were nutrient starved with Hank's balanced salt solution for the indicated time-points to induce cellular stress. Cells were stained with anti-TOM20 (red) to visualize mitochondria. Nuclei were visualized by DAPI (blue) stain and used to assess nuclear integrity as a determinant of apoptosis (D). Insets show an enlarged view of the boxed area. Arrowheads indicate cells with apoptotic nuclei. Scale bar = 10 μm. (B) Scoring of mitochondrial network morphologies based on criteria of normal, fragmented, or hyperfused for the indicated time-points and conditions. (C) Quantification of percent of cells with perinuclear mitochondrial distribution for the indicated time-points and conditions. (D) Apoptosis is expressed as percentage of cells with apoptotic nuclear morphology. Data represents mean ± SEM (*n* = 100-200 cells per group per experiment) from three independent experiments. *, $P < 0.05$ versus the corresponding CTRL at time 0; #, $P < 0.05$ versus the corresponding shCTRL condition, one-way analysis of variance with a Tukey's post hoc test.

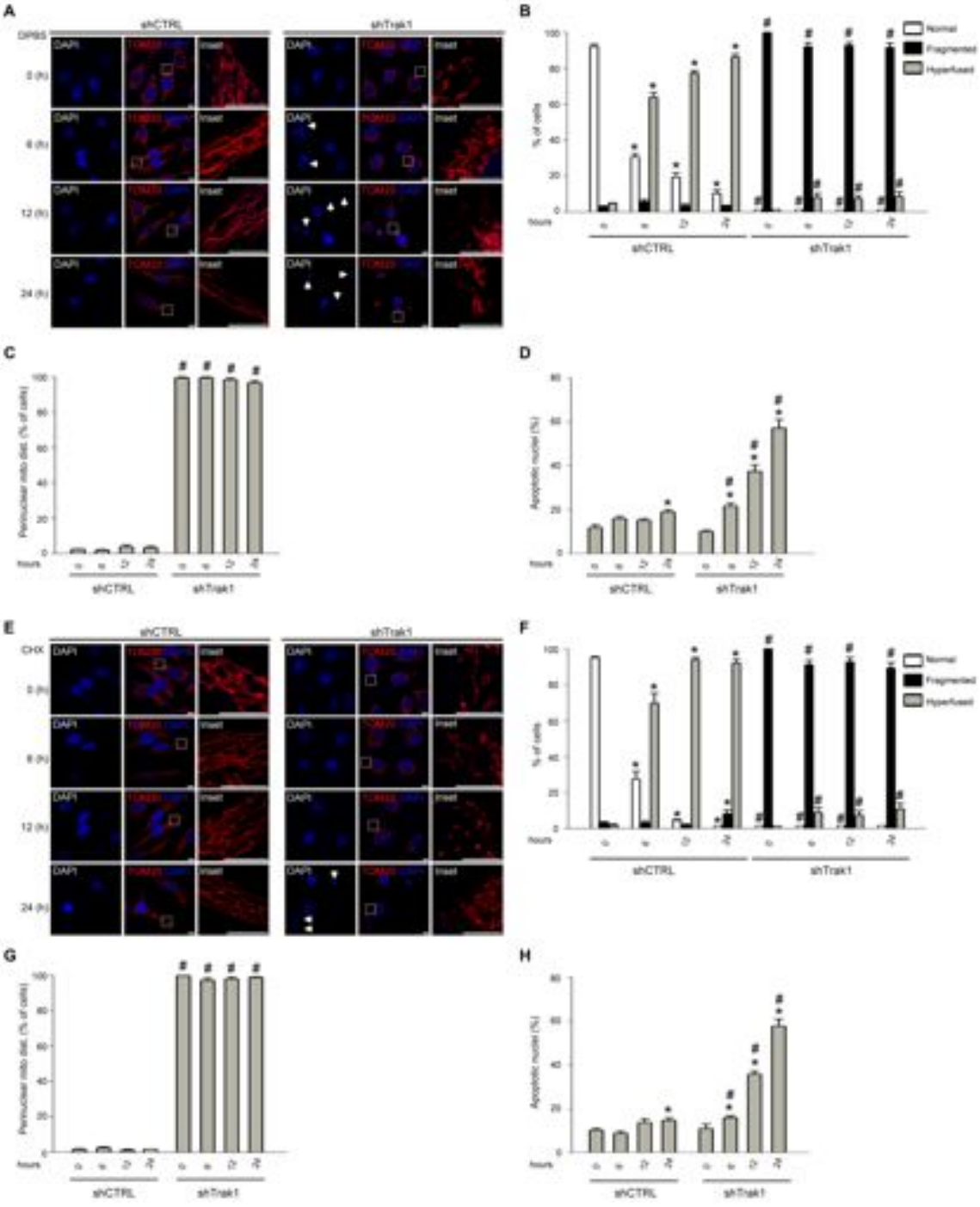

A
shCTRL
shTrak1
DPBS
CHX
DAPI
TOMM20
Inset
0 (h)
6 (h)
12 (h)
24 (h)
B
E
F
Normal
Fragmented
Hyperfused
% of cells
hours
shCTRL
shTrak1
C
G
Perinuclear mito dist. (% of cells)
D
H
Apoptotic nuclei (%)

Figure 2-12. **Mitochondrial hyperfusion occurs during cellular stress and requires Trak1.** ShCTRL and shTrak1 stable HeLa cells were either starved with DPBS (A-D) or treated with the stress stimuli cycloheximide (CHX) (E-H) for the indicated time-points. Cells were stained with anti-TOM20 (red) to visualize mitochondria. Nuclei were visualized by DAPI (blue) stain and used to assess nuclear integrity as a determinant of apoptosis (D, H). Insets show an enlarged view of boxed area. Arrowheads indicate apoptotic nuclei. Scale bar = 10 μm. Scoring of mitochondrial network morphologies based on criteria of normal, fragmented, or hyperfused for the indicated time-points and conditions for DPBS treatment (B) and CHX treatment (F). Quantification of percent of cells with perinuclear mitochondrial distribution for the indicated time-points and conditions for DPBS treatment (C) and CHX treatment (G). Apoptosis is expressed as percentage of cells with apoptotic nuclear morphology (D, H). Arrowheads indicate cells with apoptotic nuclei. Data represents mean ± SEM (n = 100-200 cells per group per experiment) from three independent experiments. *, $P < 0.05$ versus the corresponding CTRL at time 0; #, $P < 0.05$ versus the corresponding shCTRL condition, one-way analysis of variance with a Tukey's post hoc test.

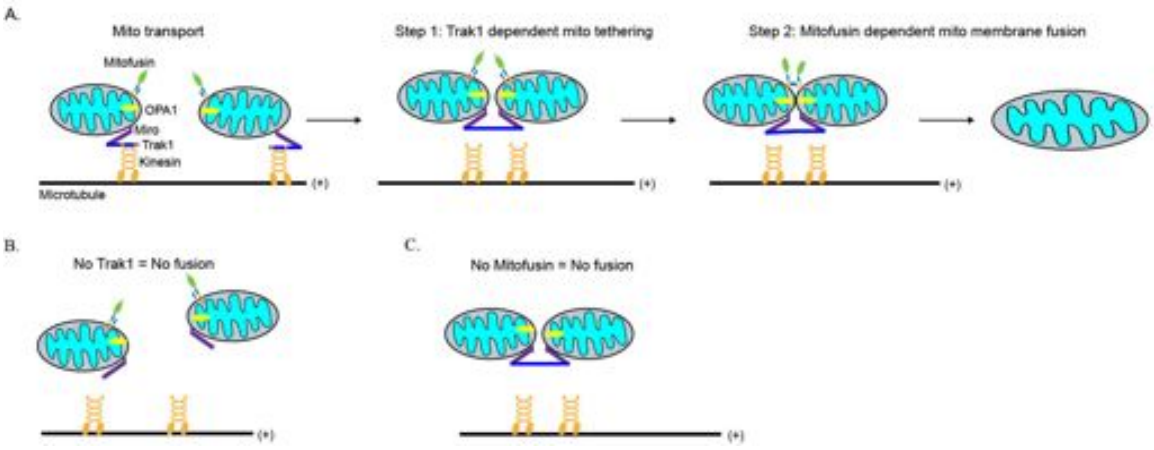


Figure 2-13. **Model: Trak1 acts in a pre-fusion step to mediate mitochondrial tethering.** (A) Trak1 is localized to the OMM and interacts with the integral OMM protein Miro and with kinesin motors to facilitate mitochondrial transport on microtubules. Mfn is localized to the OMM to regulate OMM fusion and OPA1 is localized to the IMM to regulate subsequent IMM fusion. During mitochondrial fusion, Trak1 transports two mitochondrial fusion partners to close proximity. Once mitochondrial fusion partners are appropriately positioned and close enough for fusion, Trak1 detaches from kinesin motors, and dimerizes to tether apposing mitochondria (step 1). Mfn proteins on apposing mitochondria are then able to interact for membrane fusion (step 2). (B) Depletion of Trak1 results in loss of mitochondrial transport and mitochondrial tethering thus preventing Mfns from associating and consequently mitochondria do not fuse. (C) Loss of Mfns result in mitochondria that are stuck at the pre-fusion step where apposing mitochondria are tethered but do not achieve membrane fusion.

Chapter 3:

Characterizing the role of Milton in *Drosophila* development

3.1. Abstract

The *Drosophila* protein Milton was identified as a molecular adaptor for mitochondrial transport by linking mitochondria to kinesin motors. In mammals, there are three closely related proteins to Milton that are collectively part of the HAP1 N-terminal homologous (HAPN) domain family of proteins, which include Trak1, GRIF-1, and HAP1 and all have been shown to interact with hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs), a protein component of the endocytic trafficking machinery, and regulate endosome-to-lysosome trafficking of epidermal growth factor (EGF) receptors. A role for Milton in the endocytic pathway has not been established. However, Trak1 and GRIF-1, have been shown to be required for localization of mitochondria to nerve terminals in neurons, establishing a conserved role for mitochondrial transport between flies and mammals. Furthermore, mutations in Trak1, the protein most similar to Milton, have been linked to various diseases, including hypertonia in mice and childhood absence epilepsy and gastric and colorectal cancers in humans. We found that Milton homozygous genetic nulls fail to progress beyond the first larval instar. To study the function of Milton in fly development we examined the tissue specific knockdown of Milton in the Drosophila eye, wing, and brain using the GAL4/UAS RNAi system. Loss of Milton in the fly wing results in ectopic wing veins suggesting defects in EGF receptor/MAPK signaling pathway. Additionally, we found the depletion of Milton in neurons results in reduced lifespan and locomotor climbing ability. Taken together, our observations support a role for Milton in the normal development of various fly tissues.

3.2. Introduction

Milton was first identified in a *Drosophila* genetic screen for mutations that induce synaptic transmission defects in a screen carried out in mosaic flies that were homozygous for a mutant allele in only the eyes while the rest of the body was heterozygous, in order to find genes that

could be lethal at embryonic or larval stages (Stowers et al., 2002). Flies with null mutations of Milton were blind due to loss of nerve excitation in the eye. Furthermore, mitochondria were absent in photoreceptor terminals and axons, but were abundant in the cell bodies (Glater et al., 2006; Stowers et al., 2002). Milton functions as an adaptor protein linking the mitochondrial protein Miro with conventional kinesin heavy chain to facilitate transport of mitochondria on microtubules. In mammals there are three Milton orthologues, trafficking protein, kinesin binding 1 (Trak1), $GABA_A$ receptor interacting factor-1 (GRIF-1), and Huntingtin-associated protein 1 (HAP1) (Kirk et al., 2006; Li et al., 2002; Stowers et al., 2002; Webber et al., 2008), that are collectively part of the HAP1 N-terminal homologous (HAPN) domain family of proteins. The HAPN domain proteins, including Milton, are structurally similar, containing three coiled-coil domains with the first two N-terminal coiled-coil domains encompassing the HAPN domain, the region sharing the greatest sequence homology between proteins. Milton protein shares 30%, 28%, and 25% overall amino acid identity to human Trak1, GRIF-1, and HAP1 proteins, respectively.

The mammalian Milton proteins, Trak1 and GRIF-1, have been showed to be required for mitochondrial transport in neurons, functioning as adaptors linking mitochondria through Miro to kinesin motors, suggesting that the fly and mammalian Miltons share a conserved function in mitochondrial transport (Brickley and Stephenson, 2011; van Spronsen et al., 2013). Additionally, mammalian HAPN domain proteins all have established roles outside of mitochondrial transport, as all of the mammalian HAPN domain proteins have been shown to interact directly with $GABA_A$ receptor subunits (Beck et al., 2002; Gilbert et al., 2006; Kittler et al., 2004), Hrs (Hepatocyte growth factor-regulated tyrosine kinase substrate endosomal sorting protein) (Kirk et al., 2006; Li et al., 2002; Webber et al., 2008), and regulate endocytic trafficking. A role for Milton outside of mitochondrial transport has not been established. Milton shares greatest sequences homology with Trak1 but is predicted to be the functional orthologue of all the

mammalian HAPN domain proteins in flies; therefore, may have a functional role in endocytic trafficking.

Mutations in mammalian HAPN domain proteins have been linked to various disease states highlighting the importance of understanding the function of this family of proteins. A truncation mutation in Trak1 causes recessively transmitted hypertonia in mice (Gilbert et al., 2006). In humans, the elevated expression of Trak1 is linked to gastric and colorectal cancers (An et al., 2011; Zhang et al., 2009) and a Trak1 variant is linked to childhood absence epilepsy (Chioza et al., 2009). HAP1 interacts with pathological huntingtin protein in Huntington's disease and the altered distribution of HAP1 is suggested to contribute to the pathogenesis of Huntington's disease (Li et al., 1995). *Drosophila* provides an excellent model to study normal protein function due to the high degree of conservation between humans and flies in fundamental biological and cellular pathways (Bonini and Fortini, 2003; Hirth, 2010; Pandey and Nichols, 2011).

We show that Milton is an essential protein in *Drosophila melanogaster,* as homozygous genetic nulls do not progress beyond the first larval instar. To study the function of Milton in fly development we selectively deplete Milton expression in various fly tissues using the GAL4/UAS system to overcome the lethality of null mutations at the organismal level. The *Drosophila* adult eye and wing are non-essential tissues that develop from the larval imaginal discs. The roles of several signaling pathways in wing and eye development are particularly well established (Garcia-Bellido, 1975; Garcia-Bellido and de Celis, 1992; Lloyd et al., 1998), making them a good model system for finding genetic interactions. In particular, we use the fly eye to study endocytic trafficking defects and photoreceptor neurodegeneration. Endocytic trafficking mutants can display variations in eye color due to defects in the trafficking of pigment granules (Haberman et al., 2010; Lloyd et al., 1998). Neurodegeneration in the eye can disrupt the arrangement of photoreceptor cells and phenotypically result in a rough eye morphology (Tomlinson and Ready, 1987b). Our results show that knockdown of Trak1 specifically in the eye

did not result in differences in eye color or disrupt photoreceptor patterning. In the fly wing we examine wing vein patterning to observe possible defects in signaling pathways since vein pattering is subject to EGF receptor signaling. Hyperactivation of EGF receptor signaling leads to formation of extra wing veins (Guichard et al., 1999; Wessells et al., 1999) while reduced EGF receptor signaling leads to loss of wing veins (Brunner et al., 1994; Diaz-Benjumea and Garcia-Bellido, 1990). Our results show that loss of Milton in the fly wing results in ectopic wing veins at the posterior cross vein (PCV) and at a lower frequency above the L2 and L4 longitudinal veins. This wing vein patterning is similar to what is observed upon hyperactivation of the MAPK signaling pathway (Tipping et al., 2010; Wessells et al., 1999), suggesting increase in EGF receptor signaling.

3.3. Results

3.3.1. Milton null flies display early larval lethality

To determine if Milton is an essential protein in flies, we assessed the viability of Milton null embryos. The gene encoding Milton, CG13777, is located on chromosome II. Several Milton fly lines were obtained commercially from Bloomington Stock Center that contained a P-element disruption within the *Milton* gene (Table 3-1). To generate homozygous Milton null embryos, we first balanced each of these fly lines over cyoActinGFP then crossed the heterozygotes with each other (Casso et al., 1999; Enya et al., 2014). Loss of GFP fluorescence from the cyoActinGFP balancer was used to find first instar larvae that were homozygous null for Milton. Null larvae successfully hatched from their egg cases but failed to thrive and did not progress beyond the first larval instar (Figure 3-1 A). Milton null larvae appeared unhealthy and did not actively move when observed on grape juice plates but did display withdrawal response when prodded with forceps. When homozygous null larvae were separated from their heterozygous siblings and left in uncrowded conditions they survived up to 12 days, but did not appear to progress beyond the first larval instar even though they were old enough to have attained the third larval instar (Figure

3-1). Heterozygous siblings developed normally and underwent similar developmental time points as wild-type flies. To confirm that the *Milton* gene is not expressed in the null larvae we isolated mRNA from these animals and conducted RT (reverse transcriptase) PCR. There was no detectable Milton mRNA in these larvae (Figure 3-1 B). Additionally, when different P-element heterozygous Milton null lines were crossed with each other, they failed to complement one another indicating mutations were restricted to the Milton gene. When the null alleles were crossed to a deficiency line DF(2L)ED441 that removes the section of chromosome II harboring the *Milton* gene the null alleles failed to complement and exhibited the early larval lethality phenotype. These results indicate that Milton is an essential protein and required in flies to progress beyond the first larval instar stage.

Three of the Milton P-element insertion fly lines did not exhibit larval lethality and survived into adulthood (Table 3-1). These three lines may be hypomorphic alleles or have P-element insertions that do not disrupt Milton expression. We also assessed two UAS-Milton^{RNAi} lines obtained from the Vienna Drosophila RNAi Center. When the two UAS-Milton^{RNAi} lines were crossed to a ubiquitous Tubulin-GAL4 driver the progeny from this cross were able to survive to the third larval instar but did not form pupae and no adults were seen. Very little Milton mRNA transcripts were detected from Tubulin-GAL4 > UAS-Milton^{RNAi} third instar larvae by RT-PCR (Figure 3-1 C).

3.3.2. Milton is expressed in all stages of *Drosophila* development

To assess the temporal expression of the *Milton* gene, mRNA was isolated form wild-type w^{1118} flies at various developmental stages and RT-PCR was preformed to assess the relative levels of *Milton* mRNA. Results show that *Milton* transcripts are highly expressed in the embryo and adult flies but decreases during the early larval stages and at pupation (Figure 3-2 A). *Milton* is expressed in both the adult head and body, suggesting that *Milton* expression is ubiquitously (Figure 3-2 B). Although *Milton* expression is not restricted to neurons it is enriched in fly heads

compared to bodies (Figure 3-2 B). The presence of *Milton* transcripts in the embryo could be due to maternally deposited *Milton*, which could account for the survival of Milton null flies to the first larval instar stage.

3.3.3. Milton mutants do not affect pigment granule trafficking or photoreceptor degeneration

The lethality of null mutants makes analysis of Milton in adult flies difficult. Therefore, to examine the role of Milton in adult flies we used the GAL4/UAS system to selectively drive expression of Milton RNAi in non-essential tissues (Duffy, 2002; St Johnston, 2002). The *Drosophila* compound eye is composed of a hexagonal array of 20 cells called ommatidia. Each ommatidia consists of eight photoreceptor cells (R1-R8), eight pigment cells, and four lens secreting cone cells. Each fly eye is composed of approximately 800 highly organized ommatidia arrays (Tomlinson and Ready, 1987a). Because the mammalian Milton orthologues Trak1, GRIF-1, and HAP1 proteins were shown to regulate endosome-to-lysosome trafficking of EGF receptors and interact with the endosomal sorting protein Hrs, we tested the possibility that Milton may have a similar function in endocytic trafficking. In the *Drosophila* compound eye variations in eye pigmentation are used to search for endocytic trafficking mutants (Haberman et al., 2010; Lloyd et al., 1998). Mutations that interfere with the endocytic trafficking of cargo to lysosomes and lysosome-related organelles affect pigment granules in the fly eye resulting in changes to eye color (Sevrioukov et al., 1999). Furthermore, mutations that cause neurodegeneration of the eye can be visualized at the macroscopic level as a "rough" eye phenotype (Tomlinson and Ready, 1987b). *Drosophila* EGF receptor homolog DER signaling pathway has been shown to affect ommatidial development and defects in the DER pathway have been shown to cause rough eye phenotypes (Baker and Rubin, 1989; Haberman et al., 2010).

We used the GMR (glass multiple reporter)-GAL4 diver (Freeman, 1996; Song et al., 2000) to selectively knockdown Milton in the developing fly eye. Light micrographs of adult

Drosophila compound eyes did not detect obvious differences in eye pigmentation or retinal degenerations in GMR-GAL4 > UAS-Milton^{RNAi} eyes compared to control GMR-GAL4 eyes (Figure 3-3).

3.3.4. Milton is involved in signaling pathways in the *Drosophila* wing

The adult wing is comprised of only two cell layers, one forming the dorsal wing surface and the other the ventral wing surface (Johnson and Milner, 1987). Wing veins make up the only living cells in the adult wing and functionally serve as structural supports as well as vessels for nerves and hemolymph (Johnson and Milner, 1987; Kiger et al., 2007). The veins on the wings of wild-type flies are arranged in a characteristic pattern, which consists of six longitudinal veins (L1-L6), with L2-L5 spanning the length of the wing, and two major cross veins, the anterior cross vein (ACV) and the posterior cross vein (PCV) (De Celis, 2003) (Figure 3-4 A).

The *Drosophila* adult wing develops from the larval imaginal discs (Johnson and Milner, 1987). We use two GAL4 wing drivers to reduce Milton expression in specific regions of the developing wing, MS1096-GAL4 and Engrailed-GAL4. MS1096-GAL4 is expressed in the entire wing pouch, while Engrailed-GAL4 is expressed exclusively in the posterior compartment of the wing (Akita et al., 2014). During pupation the wing discs undergoes developmental changes to produce the adult wing blade according to genetically programed steps determined in the imaginal discs. Both male and female MS1096-GAL4 > UAS-Milton^{RNAi} flies display ectopic wing veins at the PCV and in some cases above L2 and L4 longitudinal veins (Figure 3-4). The wing vein phenotype is more severe in males and males consistently also show loss of the ACV, which is not observed in female flies. The reason for these differences between male and female flies is not known. Under MS1096-GAL4 we did not observe an increase in aberrant wing vein phenotypes between flies reared at 25° C compared to those reared at 29° C, however, aberrant wing vein phenotypes become more pronounced in MS1096-GAL4; Dicer > UAS-Milton^{RNAi} flies (Figure 3-5). Dicer is an RNase III enzyme that processes RNA into siRNA to facilitate the

activation of RNA-induced silencing complex (RISC) to mediate RNAi. Overexpression of Dicer enhances RNAi activity in *Drosophila melanogaster* (Lee et al., 2004b). A similar wing vein defect is seen in Engrailed-GAL4 > UAS-Milton^{RNAi} wings (Figure 3-6). Male and female Engrailed-GAL4 > UAS-Milton^{RNAi} flies reared at 25° C displayed mild or no ectopic wing veins (Figure 3-6 B and D) but Engrailed-GAL4; Dicer > UAS-Milton^{RNAi} wing reared at 29° C had aberrant ectopic wing veins at PCV, and above L2 and L4, with males also exhibiting loss of the ACV (Figure 3-6 F and H). Control MS1096-GAL4 and Engrailed-GAL4 wings did not display aberrant wing vein phenotypes under any of these conditions. In support of the RNAi data, several genomic Milton mutants also contained ectopic wing veins (Figure 3-7). We found ectopic wing veins at the PCV in two of the Milton null lines in female flies that were heterozygous for Milton (Figure 3-7 A and B). Ectopic wing veins were not seen in male flies of the same genotype; the reason for this is not known. Two of the predicted Milton hypomorphic alleles also contained ectopic wing veins (Figure 3-7 C and D). Ectopic wing veins were found only in females heterozygous for the 22198 hypomorphic allele (Figure 3-7 C) while ectopic wing veins were found in both male and females homozygous for the 15518 hypomorphic allele (Figure 3-7 D).

Development of all *Drosophila* wing veins requires EGF receptor function. Loss of function EGF receptor mutants result in vein deletion phenotypes (Diaz-Benjumea and Garcia-Bellido, 1990) while gain of function EGF receptor alleles result in ectopic wing veins (Guichard et al., 1999; Tipping et al., 2010; Wessells et al., 1999). Activation of the EGF receptor pathway results in phosphorylation of Erk (MAPK) (Gabay et al., 1997; Schweitzer et al., 1995).

Loss of Milton leads to ectopic wing vein formation, at the PCV and at lower frequencies above the L2 and L4 longitudinal veins. Ectopic wing veins can be induced by hyperactivation of EGFR/MAPK signaling, suggesting that loss of Milton may disrupt the attenuation of this pathway, consistent with observations in human cells (Kirk et al., 2006; Webber et al., 2008).

3.3.5. Milton mutants have reduced lifespan and locomotor activity

We use Elav-GAL4, a pan-neuronal driver to reduce expression of Milton in the brain. Elav-GAL4 > UAS-Milton^{RNAi} flies are viable and develop into adulthood but show a decrease in lifespan in flies reared at both 25° C and 29° C compared to control (Elav-GAL4/+) flies (Figure 3-8). It is likely that these flies survive into adulthood due to incomplete knockdown of Milton mRNA, although we did not assess the relative levels of Milton knockdown in the brains of these flies.

Normal flies display a strong negative geotaxis response and when tapped to the bottom of a graduated cylinder will rapidly climb to the top and remain there. The loss of climbing response is used to assess behavioral manifestations of nervous system dysfunction (Feany and Bender, 2000; Pendleton et al., 2002). The Elav-GAL4 > UAS-Milton^{RNAi} flies display a premature reduction in locomotor climbing activity, as determine by a negative geotaxis assay (Figure 3-9). These data show that lifespan and climbing ability are depressed in flies with reduced levels of neuronal Milton expression.

3.4. Discussion

Receptor tyrosine kinases (RTKs) mediate diverse cellular processes, including cell fate determination during development, growth, and cell survival. Activation of RTKs results in simulation of downstream conserved RAS/MAPK pathway to ultimately affect gene expression (Lemmon and Schlessinger, 2010). RTK signaling activity is dependent on ligand availability as well as by the endocytic process (Hubbard and Miller, 2007). For signal attenuation after ligand stimulation, RTKs are endocytosis from the plasma membrane and transported to early endosomes. At the early endosome four protein complexes named the Endosomal Sorting Complex Required for Transport (ESCRT)-0, I, II, and III act sequentially to target activated RTKs to multivesicular bodies, which subsequently fuse with to lysosomes to degrade the RTKs (Rodahl et al., 2009). It has been widely reported that the major ESCRT-0 complex component

Hrs is required for attenuation of EGF receptor signaling (Jekely and Rorth, 2003; Lloyd et al., 2002) and may have a general function in RTK downregulation. The mammalian Milton orthologues Trak1, GRIF-1, and HAP1 interact with Hrs and are essential for endosome-to-lysosome trafficking of EGF receptors. The role of fly Milton in endocytic trafficking has not previously been established.

The Drosophila eye and wing are excellent systems for studying endocytic trafficking defects. GMR-GAL4 > UAS-Milton^{RNAi} flies did not appear to affect pigment granule trafficking or cause eye degeneration at the macroscopic level. It remains to be investigated if there are histological defects or defects in ommatidia arrangement at the ultrastructural level. Additionally, we use wing vein development as a sensitive genetic assay to determine if loss of Milton affects patterning and positioning of wing veins. Wing veins provide a visible readout for several signaling pathways, including Hedgehog, Wingless, Bone morphogenic protein, Notch, and EGF receptor (Blair, 2007). Loss of Milton in the fly wing results in ectopic wing veins at the PCV and at lower frequencies above the L2 and L4 longitudinal veins. While the misregulation of several signaling pathways can lead to extra or missing wing veins, the wing phenotype observed in Milton mutants resembles hyperactivation of the EGF receptor/MAPK signaling pathway. MAPK is a key downstream component in RTK signaling and the EGF receptor is the only RTK known to be involved in wing development (Guichard et al., 1999). These data represents the first report that fly Milton has a function outside of mitochondrial transport, specifically in the regulation of endocytic sorting and/or trafficking. Further experiments are necessary to clarify the relationship between Milton and expression of ectopic wing veins, particularly if Milton interacts genetically with components of the EGF receptor signaling pathway. It will also be necessary to determine the localization of Milton expression in the wing imaginal disc, as well as determine if loss of Milton results in elevated levels of activated MAPK proteins as predicted by the presence of ectopic wing veins.

We found that the selective depletion of Milton in the brain results in decreased in lifespan and reduced locomotor climbing ability. The significance of these findings are not understood at this point, but suggests that Milton is essential to normal neurological functions.

Flies have been used to study many human neurological disorders, including Huntington's disease, Alzheimer's disease, Parkinson's disease, muscular dystrophy, and neurodevelopmental disorders (Bonini and Fortini, 2003; Lessing and Bonini, 2009; Pandey and Nichols, 2011). The finding that human Trak1 is associated with neurological dysfunction and cancer and the finding that HAP1 is linked to Huntington's disease pathogenesis make understanding the function of the HAPN domain proteins an important undertaking. The possibility that similar defects in EGF receptor regulation are observed in both flies and human cell culture makes *Drosophila* an attractive model for further studies to clarify the role of Milton in the endocytic pathway. Such studies may reveal novel genetic interactions that may have counterparts in humans.

3.5. Materials and Methods

3.5.1. Fly strains

Transgenic UAS Milton RNAi lines were purchased from Vienna Drosophila RNAi Center. UAS-Milton^{RNAi} 1 is w[1118] P(GD8116)v41508 and UAS-Milton^{RNAi} 2 is w[1118]; P(GD8116)v41507/TM3. All other fly lines were obtained from Bloomington Stock Center. Milton RNAi lines were crossed to flies expressing GMR-GAL4, elav-GAL4, MS1096-GAL4, engrailed-GAL4, and tubulin-GAL4. Genomic P-element lines were purchased from Bloomington Stock center (stock numbers: 15518, 22422, 19628, 22198, 19198, 10553, 20620). Genomic P-elements lines were crossed with sp/CyoActinGFP; sb/Tm6B flies to identify homozygous null animals. W^{1118} flies was used as the control. The deficiency lines DF(2L)ED441 removes the chromosome region harboring the Milton allele.

3.5.2. RT-PCR

Embryos, larvae, pupae, and adult flies were dissolved in Trizol reagent. RNA was extracted with Trizol according to the manufacturer's instructions. Tissues were ground up using a pestle and RNA was isolated in the aqueous phase. 1ug of RNA was used for reverse transcriptase (RT) reaction with M-MLV reverse transcriptase enzyme, oligodT and dNTPs at 42° C for 52 minutes and 70° C for 15 minutes. A separate set of reactions was carried out without addition of reverse transcriptase enzyme to ensure that there is no genomic DNA contamination in the samples. 0.5ul of RT reaction was used for PCR with primers against Milton cDNA. Rp49 primers were used in a separate set of reactions as a loading control.

3.5.3 Lifespan *assay*

For lifespan assays, approximately 200 flies total of each genotype were used. Flies were kept in vials of 10-15 flies per vial and stored in a 25° C or 29° C incubator set at 12 hours light and 12 hours dark intervals. Vials were turned over every 24-48 hours and death was noted.

3.5.4. Locomotor assay

Locomotive ability of flies was assessed by negative geotaxis assay. Flies were stored at 29° C. Approximately 200 flies of each genotype were used and flies were kept at approximately 15 flies in each vial. Flies were turned over into fresh vials every 2-3 days. Before the experiment began flies were allowed to settle in a graduated cylinder for about 1 minute. When conducting the experiment, flies were tapped to the bottom of a 50 ml plastic graduated cylinder with the 25 cm line marked. The number of flies that climbed past the 25 cm mark in 15 seconds and the number of flies that climb past the 25 cm mark in 1 minute were recorded. Three trials were preformed for each time point.

P-element line	**Lethality**
Milt19628 (null 1)	Yes, not beyond fist instar
Milt 22422 (null 2)	Yes, not beyond fist instar
Milt 10553 (null 3)	Yes, not beyond fist instar
Milt 20620 (null 4)	Yes, not beyond fist instar
Milt 22198	No, survive into adulthood (hypomorph?)
Milt 19198	No, survive into adulthood (hypomorph?)
Milt 15518	No, survive into adulthood (hypomorph?)
Tubulin-GAL4 > UAS-Milton^{RNAi} 1	Yes, survive to 3rd instar but does not form pupae
Tubulin-GAL4 > UAS-Milton^{RNAi} 2	Yes, survive to 3rd instar but does not form pupae

Table 3-1. **Characterization of Milton mutant flies.** Seven P-element insertion lines were obtained from Bloomington Stock Center and 2 RNAi lines were obtained from Vienna Drosophila RNAi Center. Milton P-element lines were crossed to second chromosome balancer CyoActinGFP and then crossed with each other to generate homozygotes as determines by loss of GFP balancer. Four of the seven P-element insertion lines are lethal at early larval stages and represent genomic null alleles. Three of the seven P-element insertion lines are not homozygous lethal and may be hypomorphs. The 2 RNAi lines were crosses with Tubulin-GAL4 driver to turn on ubiquitous expression of Milton RNAi and these flies do not survive beyond the third larval stage and do not form pupae.

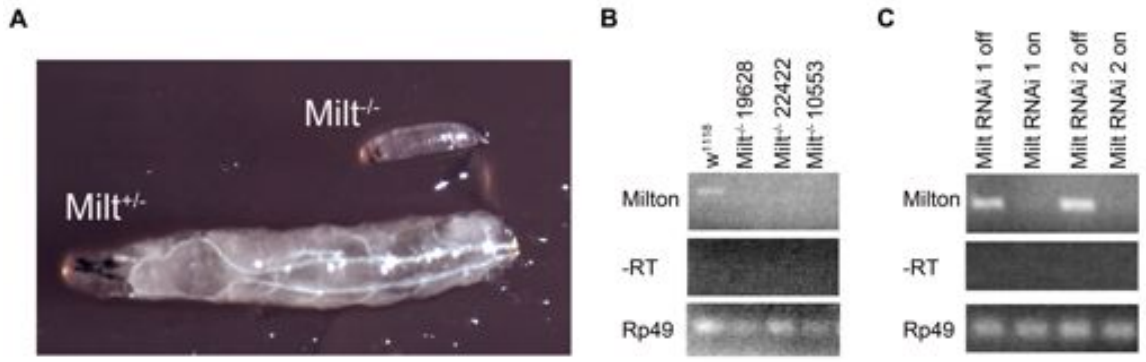


Figure 3-1. **Homozygous Milton null flies do not progress beyond the first larval instar stage.** (A) Larvae are shown at day 6 after egg laying (AEL). The homozygous nulls ($Milt^{-/-}$) do not progress beyond the first instar stage, while heterozygous ($Milt^{+/-}$) litter-mates develop normally. Eggs were laid on grape juice plates and 24 hours AEL, hatched larvae were separated based on expression of cyoActinGFP to separate homozygotes from heterozygotes. The homozygotes survive between 9-12 days but do not develop beyond the first instar stage. (B) RNA was isolated from three homozygous Milton null ($Milt^{-/-}$) lines and wild-type w^{1118} larvae at 6 days AEL and reverse transcription was preformed to generate cDNA for PCR with Milton specific primers. Milton mRNA transcripts could not be detected in Milton null ($Milt^{-/-}$) larvae but is abundant in w^{1118} larvae. (C) Milton RNAi lines crossed with Tubulin-GAL4 driver do not progress beyond the third larval instar. At the third larvae instar stage Tubulin-GAL4 > UAS-$Milton^{RNAi}$ flies contain little detectable Milton mRNA transcripts by RT-PCR while control RNAi lines not crossed with GAL4 diver at the same developmental stage contain abundant Milton transcripts. Rp49 (Ribosomal protein 49) was used as a loading control. A separate reaction with no reverse transcriptase (-RT) was performed to rule out genomic DNA contamination of samples.

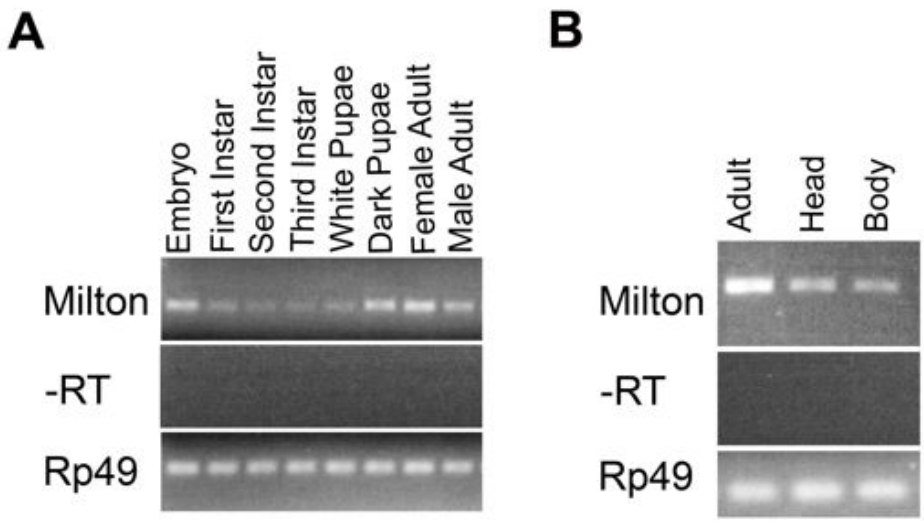


Figure 3-2. **Temporal expression of Milton transcripts through fly development.** (A) Milton mRNA transcripts are detected in all stages of fly development. Milton mRNA expression was determined by semi-quantitative RT-PCR. RNA was isolated from wild-type w^{1118} flies through different stages of development and reverse transcription was preformed to generate cDNA for PCR with Milton specific primers. (B) Milton transcripts are enriched in fly heads compared to bodies. RNA was isolated from whole adult flies, as well as just heads, and just bodies and reverse transcription was preformed to generate cDNA for PCR with Milton specific primers. Rp49 (Ribosomal protein 49) was used as a loading control. A separate reaction with no reverse transcriptase (-RT) was performed to rule out genomic DNA contamination of samples.

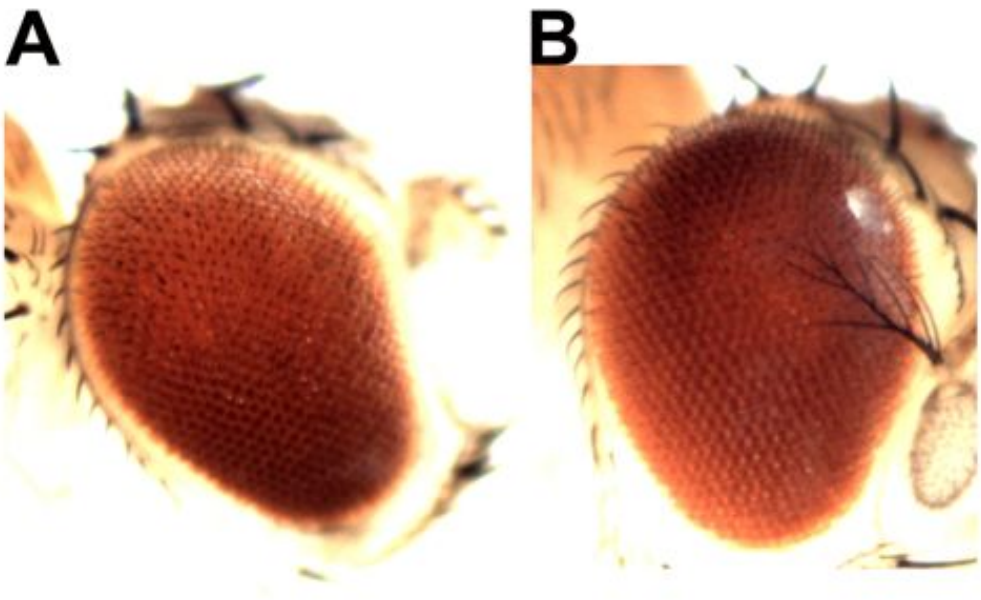


Figure 3-3. **Milton mutants do not show neurodegeneration or altered pigmentation in the eye.** Light micrographs of adult *Drosophila* eyes from (A) Control GMR-GAL4 eye and (B) GMR-GAL4 > UAS-Milton^{RNAi} eye. GMR-GAL4 > UAS-Milton^{RNAi} eyes appear morphologically normal and do not show retinal degeneration or abnormal eye pigmentation compared to control eyes. Flies were reared at 25° C.

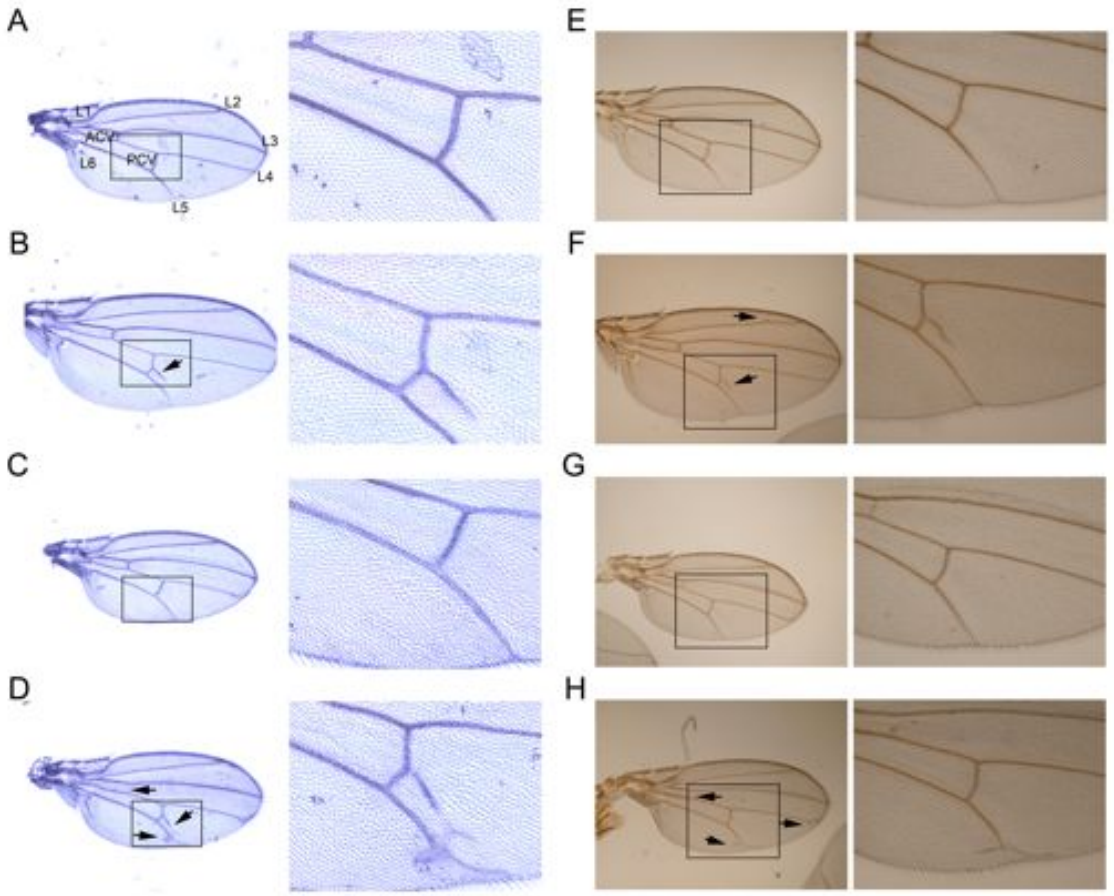


Figure 3-4. **Milton RNAi flies express ectopic wing veins under MS1096-GAL4.** (A) Female control MS1096-GAL4 wing of flies reared at 25° C show normal wing veins: L1-L6 longitudinal veins, and the transverse veins, anterior cross vein (ACV) and posterior cross vein (PCV). (B) Female MS1096-GAL4 > UAS-Milton^{RNAi} wing of flies reared at 25° C show ectopic wing veins at PCV. (C) Male control MS1096-GAL4 wing of flies reared at 25° C show normal wing veins. (D) Male MS1096-GAL4 > UAS-Milton^{RNAi} wing of flies reared at 25° C show ectopic wing veins at PCV and loss of ACV. (E) Female control MS1096-GAL4 wing of flies reared at 29° C show normal wing veins. (F) Female MS1096-GAL4 > UAS-Milton^{RNAi} wing of flies reared at 29° C show ectopic wing veins at PCV and above L2. (G) Male control MS1096-GAL4 wing of flies reared at 29° C show normal wing veins. (H) Male MS1096-GAL4 > UAS-Milton^{RNAi} wing of flies reared at 29° C show ectopic wing veins at L5 and L4 and loss of ACV. Insets show an enlarged view of boxed area. Arrows indicate aberrant wing vein phenotypes.

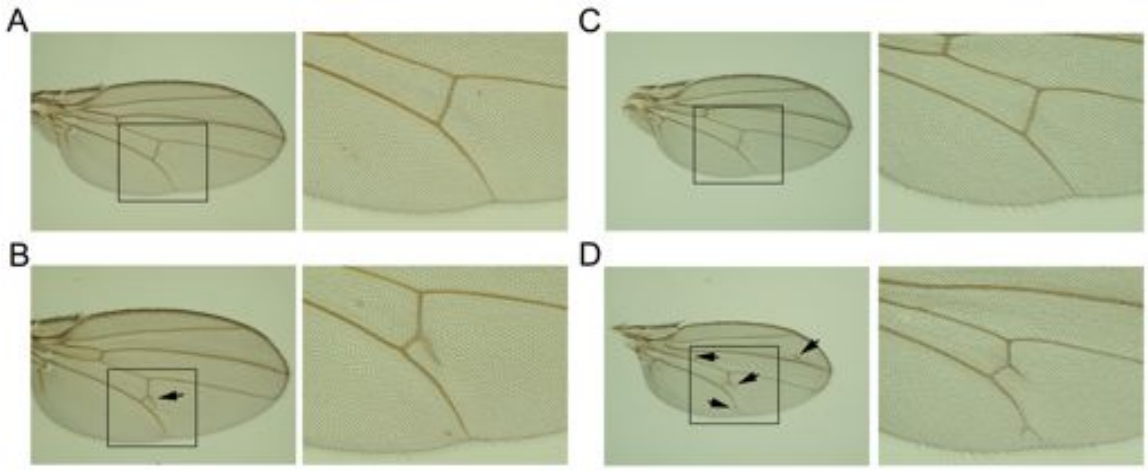


Figure 3-5. **MS1096-GAL4 > Milton RNAi flies expressing Dicer display enhanced wing vein phenotype.** (A) Female control MS1096-GAL4; Dicer wing of flies reared at 25° C show normal wing veins. (B) Female MS1096-GAL4; Dicer > UAS-Milton^{RNAi} wing of flies reared at 25° C show ectopic wing veins at PCV. (C) Male control MS1096-GAL4; Dicer wing of flies reared at 25° C show normal wing veins. (D) Male MS1096-GAL4; Dicer > UAS-Milton^{RNAi} wing of flies reared at 25° C show ectopic wing veins at PCV, L5, and L3 and loss of ACV. Insets show an enlarged view of boxed area. Arrows indicate aberrant wing vein phenotypes.

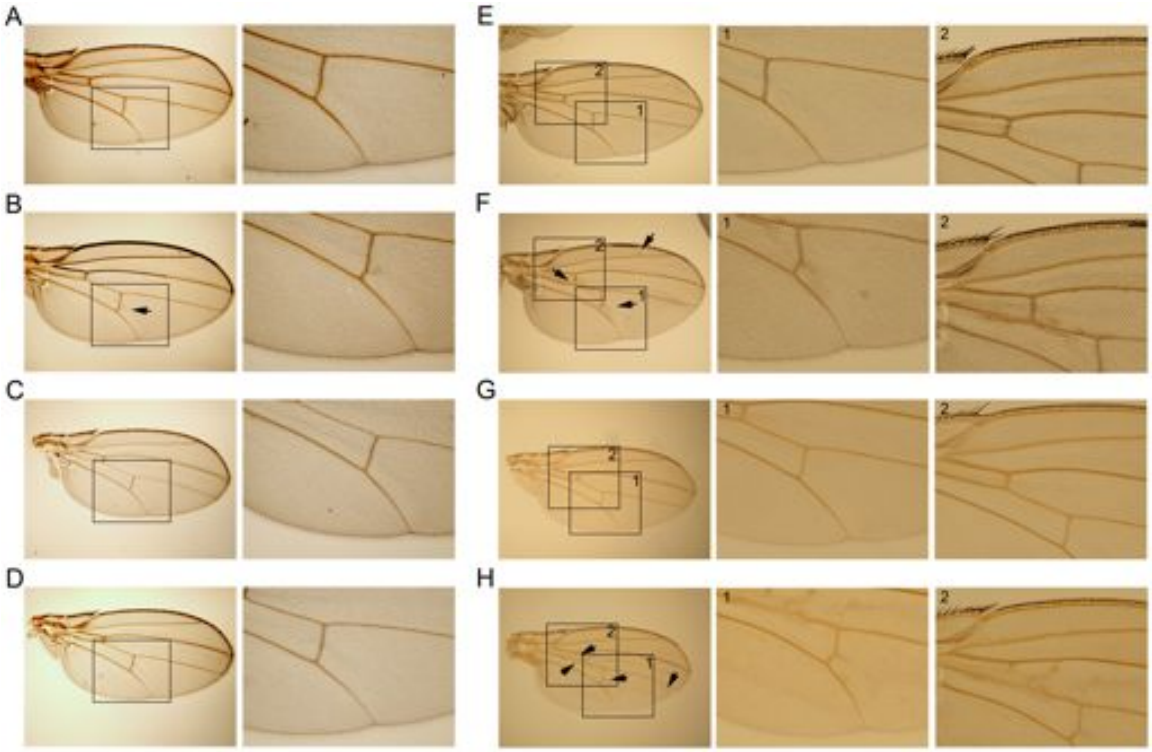


Figure 3-6. **Milton RNAi flies express ectopic wing veins under Engrailed-GAL4.** (A) Female control Engrailed-GAL4 wing of flies reared at 25° C show normal wing veins. (B) Female Engrailed-GAL4 > UAS-Milton^{RNAi} wing of flies reared at 25° C show ectopic wing veins at PCV. (C) Male control Engrailed-GAL4 wing of flies reared at 25° C show normal wing veins. (D) Male Engrailed-GAL4 > UAS-Milton^{RNAi} wing of flies reared at 25° C show normal wing veins. (E) Female control Engrailed-GAL4; Dicer wing of flies reared at 29° C show normal wing veins. (F) Female Engrailed-GAL4; Dicer > UAS-Milton^{RNAi} wing of flies reared at 29° C show ectopic wing veins at L2, L4, and PCV. (G) Male control Engrailed-GAL4; Dicer wing of flies reared at 29° C show normal wing veins. (H) Male Engrailed-GAL4; Dicer > UAS-Milton^{RNAi} wing of flies reared at 29° C show ectopic wing veins at L4, L5, and PCV and loss of ACV. Insets show an enlarged view of boxed areas. Arrows indicate aberrant wing vein phenotypes.

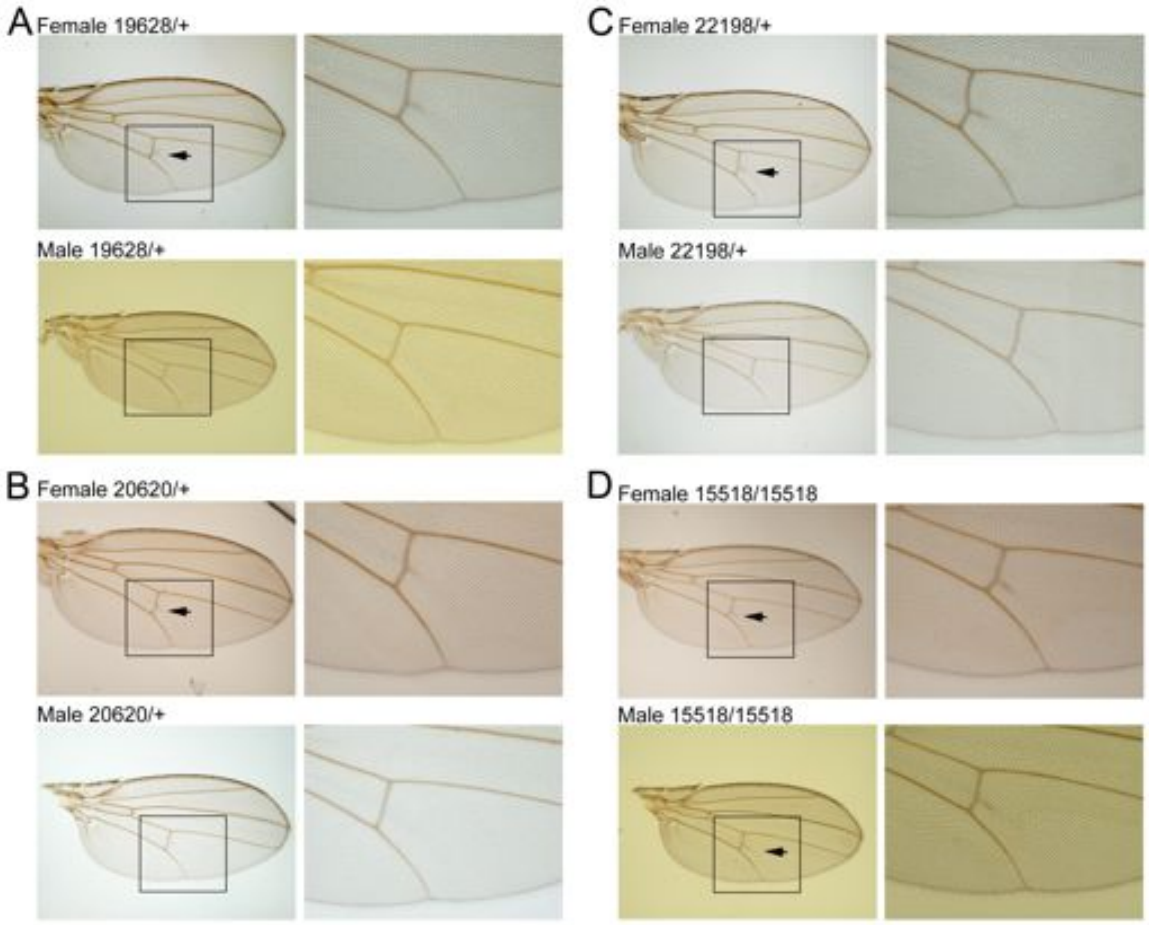


Figure 3-7. **Female genomic Milton mutants cause ectopic wing veins.** (A) Female heterozygous Milton 19628/+ wing show ectopic wing veins at PCV while male heterozygous Milton 19628/+ wing show normal wing veins. (B) Female heterozygous Milton 20620/+ wing show ectopic wing veins at PCV while male heterozygous Milton 20620/+ wing show normal wing veins. (C) Female heterozygous Milton 22198/+ wing show ectopic wing veins at PCV while male heterozygous Milton 22198/+ wing show normal wing veins. (D) Both female and male homozygous Milton 15518/15518 wing show ectopic wing veins at PCV. Flies were reared at 25° C. Insets show an enlarged view of boxed area. Arrows indicate aberrant wing vein phenotypes.

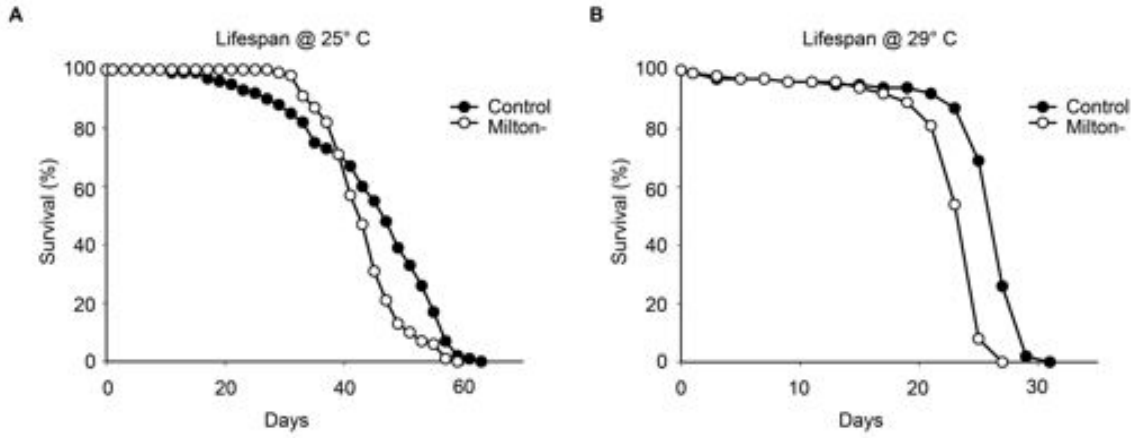


Figure 3-8. **Neuronal Milton mutants display reduced lifespan.** Control Elav-GAL4 and Elav-GAL4 > UAS-Milton^{RNAi} flies were reared at (A) 25° C and (B) 29° C and assayed for viability. Control Elav-GAL4 flies reared at 25° C display mean lifespan of 47 days and maximum lifespan of 57 days while Elav-GAL4 > UAS-Milton^{RNAi} flies display mean lifespan of 43 days and max lifespan of 51 days. Control Elav-GAL4 flies reared at 29° C display mean lifespan of 25 days and maximum lifespan of 29 days while Elav-GAL4 > UAS-Milton^{RNAi} flies display mean lifespan of 23 days and max lifespan of 25 days. Survival curves represent combined data from two independent experiments using 200 flies per group per experiment.

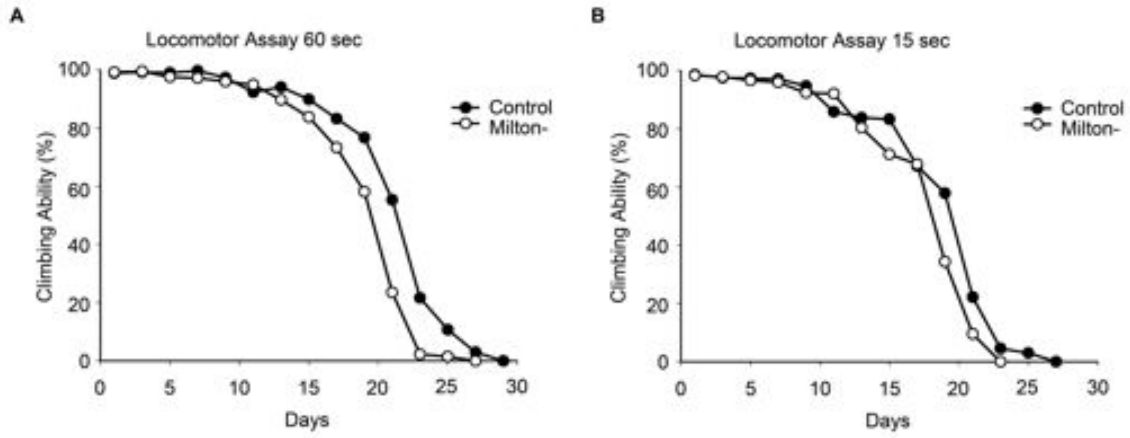


Figure 3-9. **Neuronal Milton mutants display reduced locomotor climbing activity.** Female control Elav-GAL4 and Elav-GAL4 > UAS-Milton^{RNAi} flies were reared at 29° C and assayed for locomotor climbing ability. The percent of flies able to climb to the 25 cm mark in (A) 60 sec and (B) 15 sec were recorded throughout the lifespan of the flies. Climbing ability curves represent combined data from three trials for each time-point using 200 flies per group.

Chapter 4:

Summary and Future Directions

4.1. Summary of findings

The role of Trak1 in mitochondrial transport is well established; however, whether it has a role in regulating other mitochondrial processes is not known. In this dissertation I found that Trak1 is a novel regulator of mitochondrial fusion and acts upstream of Mitofusin (chapter 2). Using cell biology and various methods of microscopy I found that Trak1 expression levels influenced mitochondrial morphology and found that Trak1 functionally interacts with Mitofusins to regulate mitochondrial fusion. The findings presented in chapters 2 demonstrate:

1. Using confocal, SIM, and TEM I showed that Trak1 depletion results in mitochondrial fragmentation and loss of mitochondria at the cell periphery, indicating that Trak1 functions in both the regulation of mitochondrial morphology as well as anterograde transport of mitochondria to the cell periphery. Since steady-state mitochondrial morphology is due to the balance between mitochondrial fusion and fission events, mitochondrial fragmentation could be due to increased fission or loss of fusion. To clarify the role of Trak1 in mitochondrial morphology I showed that knockdown of Trak1 reduces the rate of mitochondrial fusion compared to control cells using live cell time-lapse confocal imaging. In support of the role of Trak1 in promoting fusion the overexpression of Trak1 resulted in hyperfused mitochondria that were elongated and enlarged, as shown by confocal, SIM, and TEM.
2. I found that Trak1 acts upstream of Mitofusins and that Trak1 is required for Mitofusin mediated mitochondrial fusion. In Trak1 depleted cells, the overexpression of Mitofusins could not rescue mitochondrial fragmentation to restore mitochondria to tubular morphology. Furthermore, in Mitofusin null MEFs the overexpression of Trak1 resulted in mitochondrial clustering with apposing mitochondrial membranes in close proximity. I interpret these results as Trak1 being required to tether adjacent mitochondria in a pre-fusion step in order for Mitofusins on adjacent mitochondria to interact and drive mitochondrial membrane merging (Figure 2-12). In the absence of Mitofusins,

mitochondrial fusion does not occur and Trak1 overexpression results in mitochondria that are "stuck" in a tethered state.

3. I provide a novel mechanism for Trak1 associated hypertonia pathogenesis and show that the hyrt mutation of Trak1 is partially mistargeted away from mitochondria to the cytosol, thus affecting the ability of Trak1 hyrt to regulate mitochondrial fusion. Impaired mitochondrial fusion in hyrt mutants could result in impaired mitochondrial functions, such as ATP production or calcium buffering, leading to hypertonia pathogenesis.
4. Finally, I show that Trak1 is required for stress-induced mitochondrial hyperfusion and that loss of mitochondrial fusion during cellular stress increases susceptibility to cell death. This data provides a functional link between Trak1 and cellular health under stress conditions.

Trak1 is part of the HAPN domain family of proteins and our lab has previously shown that Trak1 along with the other HAPN domain family members, GRIF-1 and HAP1, interact with Hrs endosomal sorting protein and regulates the endosome-to-lysosome trafficking of EGF receptors (Kirk et al., 2006; Li et al., 2002; Webber et al., 2008). In the fruit fly *Drosophila melanogaster* there is a single HAPN domain protein, Milton (Stowers et al., 2002). Although Milton is most similar to Trak1 in sequence homology it is predicted to be the functional orthologue of all three mammalian HAPN domain proteins. A role for Milton in mitochondrial transport is well established (Glater et al., 2006; Stowers et al., 2002), however, a role for Milton in endocytic trafficking has not been established. In this dissertation, I investigate the role of Milton in fly development and specifically assess the ability of Milton mutants to alter endocytic trafficking in the fly eye and wing (chapter 3). Establishing a role for Milton in endocytic trafficking will clarify the conserved role of Milton as a dual functioning protein and will allow for the identification of novel genetic interactions using the fruit fly as a model. I found that loss of Milton in the fly wing resulted in an ectopic wing vein phenotype, resembling that of EGF receptor/MAPK hyperactivation. This finding is the first to demonstrate that loss of Milton may

disrupt the attenuation of the EGF receptor/MAPK pathway, consistent with observations with Trak1 in human cells. The findings presented in chapters 3 demonstrate:

1. I characterized several commercially available Milton mutant flies that harbor P-element insertions within the Milton gene to disrupt Milton expression. I found several of the P-element insertion lines were true null alleles and did not express Milton specific mRNAs when assessed by RT-PCR. These Milton null alleles proved to be lethal at the first larval instar when homozygous. Two commercially available RNAi lines were lethal at the third larval instar stage when crossed with a ubiquitous Tubulin-GAL4 driver. Survival of the Tubulin-GAL4 > UAS-Milton^{RNAi} lines to the third larval instar is likely due to incomplete knockdown of Milton expression.
2. I found Milton to be widely expressed in wild-type flies and Milton expression was found in all stages of development and in various fly tissues, indicating ubiquitous expression similar to Trak1 in mammals.
3. The knockdown of Milton in the fly compound eye did not result in changes to eye pigmentation or appearance of ommatidia degeneration, indicating that Milton may not affect the endocytic trafficking of eye pigment granules or the patterning of ommatidia during development, although more studies are needed to confirm these findings.
4. I found that knockdown of Milton in the fly wing using two wing specific GAL4 drivers resulted in ectopic wing vein formation. Ectopic wing veins were also found in several heterozygous genomic Milton null alleles. The ectopic wing vein pattern most resembled that of hyperactivation of EGF receptor/MAPK signaling, suggesting that Milton, like Trak1 in mammals, may regulate the degradation of activated EGF receptors by endosome-to-lysosome trafficking.
5. I found that knockdown of Milton specifically in neurons using the pan-neuronal Elav-GAL4 driver resulted in a decrease in lifespan and locomotor climbing ability, suggesting that Milton is important for neurological functions in flies.

These finding contribute to a better understanding of the role of Trak1 and related protein Milton in important biologically processes that contribute to cellular health and homeostasis, as well as in disease pathogenesis. In this section, I will discuss the implications of these findings as well provide suggestions for future experiments.

4.2. Future directions

Many questions remain regarding the specific function of Trak1 in the dual regulation of endocytic trafficking and mitochondrial dynamics. The fact that the Trak1 orthologue, Milton, is essential in flies and that in mammals, Trak1 mutations are associated with disease states highlights the significance of Trak1 in cellular homoeostasis and organismal health. Furthermore, no overarching mechanistic understanding has been reached to explain how Trak1 can regulate two separate cellular pathways.

4.2.1. Does loss of Trak1 affect the function of mitochondria?

Depletion of Trak1 results in mitochondrial fragmentation (chapter 2) and fragmented mitochondria are often accompanied by mitochondrial and cellular dysfunction (Chen et al., 2005). In particular, fragmented mitochondria are observed in several neurodegenerative diseases and may contribute to disease pathology (Costa et al., 2010; Irrcher et al., 2010; Knott et al., 2008; Reddy and Shirendeb, 2012; Wang et al., 2008). Although mitochondrial fragmentation in and of itself does not lead to compromised mitochondrial functions, the loss of mitochondrial fusion prevents unhealthy or damaged mitochondria from repairing itself through fusion with healthy mitochondria and consequently leads to the accumulation of damaged mitochondria over time. Damaged mitochondria release high levels of Ca^{2+} and cytochrome c to trigger apoptosis (Parsons and Green, 2010); therefore, repair of damaged mitochondria through fusion is critical to cell survival.

Fragmented mitochondria as a result of Trak1 depletion are more sensitive to cellular stress resulting in significant cell death, while control cells were able to undergo stress-induced hyperfusion and were spared from cell death. This finding suggests that Trak1 is cell protective at least during cellular stress conditions. Further studies are needed to investigate whether mitochondrial fragmentation seen in Trak1 depleted cells leads to impaired mitochondrial functions under normal growth conditions. In mouse embryonic fibroblasts (MEFs) that are genetic knockouts for either Mfn1 or Mfn2 mitochondria are severely fragmented but do not display gross defects in respiration and only a small subset of individual mitochondria loose their membrane potential (Chen et al., 2003a). However, MEFs that lack both Mfn1 and Mfn2 show severe defects in cellular respiration and dissipation of mitochondrial membrane potential (Chen et al., 2005). Mfn1 or Mfn2 deficient MEFs retain a low level of mitochondrial fusion while double knockouts completely lack mitochondrial fusion (Chen et al., 2005; Chen et al., 2003a), suggesting that mitochondrial fragmentation itself may not lead to a major decline in mitochondrial functions, while a complete loss of fusion activity leads to widespread decline in mitochondrial functions. Furthermore, *in vivo* rates of fusion and fission greatly exceed what is needed to maintain mitochondrial tubular morphology (Olichon et al., 2003), suggesting that the constant fusion and fission events are needed to maintain mitochondrial homeostasis through the efficient distribution of mtDNA and proteins throughout the mitochondrial network.

To address the possibility that Trak1 is required to maintain mitochondrial functions, studies will be needed to probe mitochondrial membrane potential, ATP production, calcium handling, and cytochrome c release in Trak1 knockdown cells. It is likely that Trak1 knockdown cells retains some fusion activity, as in my shTrak1 cells I observe some fusion activity using mito-Dendra assay; therefore, it is possible that no major decline in mitochondrial function will be observed under normal growth conditions. In that case, it may be necessary to generate genomic Trak1 knockout cells using mouse genetics, such as those used to produce the Mfn1, Mfn2, and Mfn 1 and 2 knockout MEFs (Chen et al., 2005; Chen et al., 2003a). If Trak1 is

required for fusion then Trak1 knockout MEFs should also display widespread decline in mitochondrial respiratory functions and dissipation of mitochondrial membrane potential.

4.2.2. How does Trak1 regulate two separate cellular pathways?

Trak1 is localized significantly to two separate subcellular compartments, mitochondria and early endosomes, and functions in two separate cellular pathways, mitochondrial dynamics and endosome-to-lysosome trafficking. No overarching mechanistic understanding has been reached to explain how Trak1 can be targeted to and regulates two separate cellular pathways or how Trak1 can selectively discriminate between these subcellular compartments. For example, it is not known if Trak1's role in endosomal trafficking is linked to its function as an adaptor for kinesin mediated transport of mitochondria on microtubule filaments. One possibility is that Trak1 functions as an adaptor for the microtubule based transport of early endosomes and by this mechanism Trak1 mutants inhibit endosome-to-lysosome trafficking rather than affecting the Hrs-mediated endosomal sorting of cargo proteins, the mechanism favored by Webber et al (Webber et al., 2008). However, while the knockdown of Trak1 has been shown to inhibit the microtubule based transport of mitochondria, resulting in immobile mitochondria, the knockdown of Trak1 did not affect the distribution or motility of early endosomes, so it is possible that Trak1's role in the endosomal pathway is unconnected to its role in kinesin mediated transport. To potentially sort out the contributions of Trak1 to these separate pathways, the role of Trak1 in each pathway would have to be evaluated separately.

Trak1 lacks mitochondrial targeting sequence, an FYVE domain for endosomal targeting, or a transmembrane domain; therefore, the mechanism of Trak1 targeting to the early endosome and mitochondria are dependent on interactions with proteins localized to these organelles. Localization of Trak1 to early endosomes is dependent on interaction with Hrs and removal of the Hrs binding region of Trak1 (residues 354-953) abolished early endosome localization (Webber et al., 2008). The association of Trak1 to KHC is direct and requires the KHC binding region of

Trak1 (residues 201-306) (Smith et al., 2006; van Spronsen et al., 2013). Trak1 localization to mitochondria is predicted to require Miro binding based on co-immunprecipitation experiments (Fransson et al., 2006; MacAskill et al., 2009a; Macaskill et al., 2009b; Ogawa et al., 2014; Pekkurnaz et al., 2014; Wang and Schwarz, 2009; Weihofen et al., 2009) and the mitochondria targeting sequence of Trak1 is attributed to amino acids 599-800 (Koutsopoulos et al., 2010). However, studies have not been done to show that Trak1$^{599\text{-}800}$ conferred Miro binding, or that Trak1 localization to mitochondria was strictly dependent on interaction with Miro. Furthermore, one study found that Miro with deletion of its transmembrane domain (MiroΔTM) was strictly cytosolic but overexpression of Trak1 could target MiroΔTM to mitochondria, suggesting that Trak1 localization to mitochondria is not strictly Miro dependent (Koutsopoulos et al., 2010). Our studies revealed that overexpression of Trak1 resulted in targeting of exogenous Trak1 proteins to outer mitochondrial membrane (OMM), suggesting that there is either a large population of unbound endogenous Miro proteins at the OMM or Trak1 targeting to mitochondria is not exclusively dependent on Miro binding. It is possible that additional unknown mitochondrial proteins or even mitochondrial lipids could contribute to Trak1 mitochondrial localization. Therefore, additional studies are needed to clarify the Trak1/Miro interaction, Miro binding region of Trak1, and whether Trak1 targeting to mitochondria is strictly dependent on interaction with Miro. *In vitro* binding assays with purified recombinant proteins are needed to assess the direct binding of Trak1 with Miro, and potentially other OMM proteins, similar to what has been done by Webber et al. to show Trak1 and Hrs directly and specifically interact with each other. Additional deletion and co-immunoprecipitation studies are needed to define the minimal residues required for Trak1 interaction with various endocytic trafficking and mitochondrial dynamics proteins as well as to generate point-mutations that specifically impair interaction with a specific protein without affecting interactions with other proteins. Furthermore, no one has examined how various Trak1 deletion mutants affect targeting to both organelles simultaneously. The subcellular localization of various deletion mutants should be evaluated to compare the

localization to the early endosome, mitochondria, and the cytosol in order to determine which deletion mutants yield more or less targeting to these locations. Subcellular fractionation can also be utilized to compare the localization of various deletion mutants to these sites compared to Trak1 WT.

After the minimal targeting sequences to each organelle have been identified it will be possible to create an endosome specific Trak1 isoform and a mitochondria specific Trak1 isoform to be able to assess the specific function of Trak1 at each subcellular location. Expression of each isoform should be done in a Trak1 knockdown or Trak1 knockout cell line and assays should be done in these cells to address endocytic trafficking, mitochondrial morphology, mitochondrial fusion/fission, mitochondrial transport, and mitochondrial functions to clarify the specific contribution of Trak1 at each organelle. Creation of a Trak1 mutant abolishing Trak1 kinesin binding will also help clarify the role of kinesin mediated transport for Trak1 regulation of endocytic trafficking as well as to be able to separate the transport and fusion functions of Trak1 in mitochondrial dynamics. Lastly, expression of the endosome specific and mitochondria specific isoforms in a Trak1 hyrt background, such as neurons or MEFs derived from Trak1 hyrt mouse model may help elucidate the pathogenic mechanisms of the Trak1 hyrt mutation.

4.2.3. What is the specific mechanism of Trak1 mediated mitochondrial tethering?

Our lab (data not shown) as well as others have shown that Trak1 self interacts to form homo-dimers (Koutsopoulos et al., 2010). The Trak1 dimerization region is predicted to encompass amino acid residues 1-380 (Koutsopoulos et al., 2010). This region also encompasses the kinesin-binding region of Trak1 (amino acid residues 201-306) (Smith et al., 2006; van Spronsen et al., 2013). Based on the data presented in chapter 2, we hypothesize that Trak1 mediates the transport of two mitochondrial fusion partners to close proximity through interaction with kinesin and microtubule based transport. Once mitochondrial fusion partners are appropriately positioned and close enough to induce fusion, Trak1 detaches from kinesin and

dimerizes with Trak1 on the apposing mitochondria to tether the mitochondrial fusion partners and to allow Mitofusins on apposing mitochondria to interact and drive member merging. To test this hypothesis a critical experiment is to create Trak1 point mutations that prevent KHC interaction but does not affect Trak1 dimerization to separate the transport and tethering functions. The converse experiment will also be needed in which point mutations are generated that inhibit Trak1 dimerization but do not affect interaction with KHC to show that tethering capability and consequently fusion is lost or impaired while transport is preserved. These experiments may or may not be possible depending on ability to generate these specific point mutations that affect one function but not the other.

4.2.4. Can Trak1 be used therapeutically, such as to rescue the excessive mitochondrial fragmentation and neuronal dysfunction seen neurodegenerative diseases?

Mitochondrial fragmentation is observed in several common neurodegenerative disorders including, AD, PD, HD, and ALS (Bossy-Wetzel et al., 2008; Exner et al., 2007; Knott et al., 2008; Reddy and Shirendeb, 2012; Wang et al., 2008). Although mitochondrial fragmentation and dysfunction are characteristic of many neurodegenerative disorders, it is not known if mitochondrial fragmentation is causal to these diseases or what might trigger the pathological activation of mitochondrial fission in theses diseases. However, loss-of-function mutations in genes that encode mitochondrial fusion proteins have been shown to cause neurodegenerative disease. Mutations in Mfn2 cause Charcot-Marie-Tooth subtype 2A (CMT2A), a peripheral neuropathy characterized by axonal degeneration of sensory and motor neurons (Kijima et al., 2005; Zuchner et al., 2004). Several of the Mfn2 mutations linked to CMT2A have been shown to impair mitochondrial fusion, while other mutations in Mfn2 may disrupt mitochondrial trafficking (Detmer and Chan, 2007a). Mutations in OPA1 cause autosomal dominant optic atrophy, which is characterized by progressive vision loss and degeneration of the optic nerve and retinal ganglion cells (Carelli et al., 2004).

There is evidence that restoration of mitochondrial tubules and reduction in mitochondrial fission may alleviate some aspects of neuronal dysfunction and restore mitochondrial health. Several studies have shown that expression of Mitofusins was cell protective during oxidative stress (Barsoum et al., 2006; Jahani-Asl et al., 2007) or could rescue mitochondrial fragmentation and cellular dysfunction induced by disease-linked mutations (Detmer and Chan, 2007a; Wang et al., 2009). For example, in HeLa cells the overexpression of mutant huntingtin (Htt74Q) results in mitochondrial fragmentation, reduced ATP production, and increased cell death, however, expression of Mfn2 or dominant-negative Drp1 restores mitochondrial morphology and ATP levels, and prevented cell death (Wang et al., 2009). It would be important to determine if Trak1 expression can rescue the fragmentation and/or trafficking defects seen in neurodegenerative disease linked gene mutations to restore mitochondrial morphology and function. To start, the disease associated mutant proteins can be expressed in cell culture models and attempted rescue with expression of Trak1 WT can be preformed to assess the restoration of normal tubular mitochondrial morphology and ATP levels.

4.2.5. Does Trak1 regulate other aspects of mitochondrial dynamics such as fission or mitophagy?

Although other groups have shown that Trak1 is required for mitochondrial transport (Brickley et al., 2005; Brickley and Stephenson, 2011; Glater et al., 2006; van Spronsen et al., 2013) and my data clearly establishes that Trak1 directly regulates mitochondrial fusion, it remains to be tested if Trak1 is involved in the regulation of other processes of mitochondrial dynamics, such as mitochondrial fission and/or mitophagy. It is possible that loss of Trak1 may increase Drp1 fission activity to induce mitochondrial fragmentation. To address the possibility that Trak1 acts as a repressor of fission we could inhibit mitochondrial fission in Trak1 knockdown cells to determine if this could restore mitochondria to tubular morphology. Drp1 proteins directly mediate the mitochondrial fission process and a dominant-negative allele of Drpl,

Drp1-K38A blocks mitochondrial fission when expressed (Smirnova et al., 2001). If Trak1 is strictly required for fusion, then Drp1-K38A would have no effect and mitochondria would remain fragmented, however, if Trak1 regulates Drp1 activity than expression of Drp1-K38A could rescue mitochondrial fragmentation in Trak1 depleted cells.

Mitochondrial dynamics also encompasses the process of mitophagy, a form of autophagy by which damaged mitochondria are selectively degraded (Ashrafi and Schwarz, 2013). During mitophagy, damaged mitochondria must be separated from the healthy mitochondrial network; therefore, mitophagy may be closely linked to other aspects of mitochondrial dynamics including fusion/fission and transport. Studies in yeast indicate that the yeast homolog of Drp1 is essential to mitophagy (Kanki et al., 2010), while studies in mammals have shown that mitochondrial hyperfusion prevents autophagic degradation of mitochondria (Twig and Shirihai, 2011). For example, under certain types of cellular stress or starvation conditions mitochondria hyperfuse to maintain ATP levels and are protected from autophagic degradation in a process called stress-induced mitochondrial hyperfusion (Rambold et al., 2011; Tondera et al., 2009).

Mitochondrial transport also likely plays a key role in mitophagy, particularly in the transport of damaged mitochondria to the cell body from distal neuronal regions, as well as to arrest mitochondrial transport during later steps of mitophagy to prevent the transport and fusion of damaged mitochondria with healthy mitochondria. In mammals, PINK1 and parkin mediate the elimination of damaged mitochondria through mitophagy. PINK1 and parkin have been found in a protein complex with Miro and Trak1 in depolarized mitochondria (Weihofen et al., 2009) and PINK1 phosphorylates Miro during mitophagy to induce its proteasomal degradation (Wang et al., 2011), however, it is not known if Trak1 is also targeted for degradation during mitophagy. Because Trak1 is required for both mitochondrial transport and fusion it stands to reason that Trak1 could play an important role during mitophagy. To directly assess the role of Trak1 in mitophagy, several experiments can be preformed. Mitophagy assays can be conduced in stable shTrak1 and shCTRL cells in which the entire cellular population of mitochondria are

depolarized using the chemical uncoupler CCCP (Narendra et al., 2008) and imaged under fluorescent microscopy to observer recruitment of PINK1/parkin and the autophagy marker LC3 to mitochondria at early time points and loss of mitochondria through mitophagy at later time points (Narendra et al., 2008). Damage of a subset of mitochondria can also be achieved using photoirradiation (Kim and Lemasters, 2011) or mitochondrial DNA damage (Suen et al., 2010) in shTrak1 and shCTRL cells to look for the selective removal of these damaged mitochondria using imaging techniques. Several possibilities exist as to how loss of Trak1 might affect mitophagy, one possibility is that loss of Trak1 and the consequent mitochondrial fragmentation could make mitochondria more susceptible to mitophagy. Therefore, we may observe increased mitophagy compared to control cells. Another possibility is that that loss of Trak1 mitochondrial transport or fusion functions may affect the efficient recruitment of the mitophagy machinery or inhibit mitophagy response; therefore, we may observe impaired mitophagy compared to control cells.

4.2.6. What is the pathogenic mechanism of Trak1 hyrt associated hypertonia?

The pathogenic mechanism of the Trak1 hyrt mutation remains elusive. A previous study in our lab did not identify differences in early endosome localization, interaction with Hrs, or alter endocytic trafficking between Trak1 WT and Trak1 hyrt (Webber et al., 2008). Studies in the hypertonia mouse model with recessive Trak1 hyrt mutation showed reduced expression levels of $GABA_A$ receptors in the CNS but co-immunoprecipitation experiments from wild-type and hyrt mice did not detect differences in binding between Trak1 and $GABA_A$ receptors in these mice (Gilbert et al., 2006). It is not known how the Trak1 hyrt mutation affects the endocytic trafficking or expression of $GABA_A$ receptors. Another possibility is that the Trak1 hyrt mutation impairs the post-translational modification of Trak1 by OGT. The OGT enzyme binds Trak1 at resides 639-859 to glycosylate Trak1 at several sites to facilitate inhibition of mitochondrial transport in response to elevated glucose levels (Iyer and Hart, 2003; Pekkurnaz et al., 2014); therefore, the Trak1 hyrt mutation, which results in Trak1 protein truncation at amino acid 824

and deletes several glycosylation sites could affect the post-translational regulation of Trak1 in mitochondrial motility, although this has not been directly tested. Additionally, the functional consequences of Trak1 glycosylation by OGT for endocytic trafficking are not known.

My studies showed that the Trak1 hyrt mutation displayed altered mitochondrial localization compared to Trak1 WT and was less effective at mitochondrial fusion. This suggests that the missing amino acid residues in Trak1 hyrt confers efficient targeting to mitochondria, although it is not essential for mitochondrial targeting, since Trak1 hyrt protein is still largely targeted to mitochondria and can partially rescue the mitochondrial defects seen in shTrak1 cells. To assess the effect of Trak1 hyrt it would be necessary to obtain the hyrt mouse model or generate a cell culture model in which endogenous Trak1 is depleted but the hyrt mutation is stably expressed at levels comparable to endogenous Trak1. Using either of these two models it would be important to address the morphology and health of mitochondria, as well as to assess if the hyrt mutation impaired mitochondrial transport. It would be particularly useful to address these questions in a neuronal model to look for altered distribution or trafficking of mitochondria and impaired neuronal function and health, since hypertonia is primarily a movement disorder that may result from defects in motor pathways

The mechanism by which Trak1 hyrt is partially mistargeted to mitochondria remains unknown. The Miro binding region of Trak1 as well as how Trak1 is targeted to mitochondria remains unclear and it is also unknown how the hyrt truncation affects Trak1 protein folding and consequently its ability to interact with other proteins. The hyrt mutation is not suspected to interfere with kinesin binding or dimerization, which is supported by my findings that the hyrt mutation could partially rescue mitochondrial fragmentation in a Trak1 knockdown stable cell line and overexpression of Trak1 hyrt induced mitochondrial hyperfusion, albeit less efficiently than Trak1 WT. Additional studies are needed to address these questions and are dependent first on our understanding of the mechanisms of Trak1 WT as discussed in the above sections.

One potential experiment that could be carried out is to perform mass spectrometry studies to identity proteins that may differentially interact with Trak1 hyrt compared to Trak1 WT, specifically altered protein interactions at the mitochondria and early endosomes. Briefly, epitope-tagged Trak1 can be exogenously expressed in cells and mitochondria or endosomes can be purified from cell lysates. Tagged-Trak1 WT and Trak1 hyrt can be pulled down in the resulting mitochondria or early endosome fractions and subjected to mass spectrometry and verification of candidate proteins can be achieved though co-immunoprecipitation experiments. The identification of additional Trak1 binding partners may be achieved and possibly the finding of pathological interactions of Trak1 hyrt with other proteins may provide clues to the mechanism of Trak1 associated hypertonia.

4.2.7. Does Milton share functional homology with Trak1 in flies?

In my dissertation I found that loss of Milton resulted in larval lethality and ectopic wing veins. The ectopic wing vein pattern most resembled that of hyperactivation of EGF receptor/MAPK signaling, suggesting that Milton, like Trak1 in mammals may regulate the degradation of activated EGF receptors by endosome-to-lysosome trafficking. Further studies are needed to validate these finding including whether phospho-ERK is increased in Milton null wing discs and if Milton interacts genetically with components of the EGF receptor signaling pathway. Immunocytochemistry can also be done to identify the subcellular localization of Milton and to establish colocalization between Milton and fly Hrs. Studies are also needed to determine if human Trak1 can functionally rescue lethality seen in Milton null mutants. Transgenic fly lines can be created to express Trak1 WT or Trak1 hyrt under the UAS promoter, that way expression of these transgenes can be turned on using either a ubiquitous-GAL4 driver or a tissue specific driver. Transgenic flies that express Trak1 hyrt in Milton null flies can be used to assess the development of neuropathological phenotypes or hypertonia. These transgenic flies can also be used to test the ability of Trak1 hyrt to rescue larval lethality and ectopic wing veins. To

determine if Trak1 hyrt expression in Milton null flies contributes to hypertonia, locomotor behavior can be tested using walking and climbing assays (Koh et al., 2004; Pendleton et al., 2002), assessment of neurological defects such as abnormal leg or wing twitching can be used to determine if Trak1 hyrt enhances muscle contractions (Koh et al., 2004), and electrophysiological recordings can be done to measure motor unit activity (Budnik et al., 1990; Friesen and Wyman, 1980; Umbach et al., 1994). Additionally, experiments will also be needed to examine Milton null larvae for mitochondrial fusion defects to determine if Milton also functions in mitochondrial fusion. The prediction would be that Milton null flies would contain fragmented mitochondria.

4.3. Final words

Trak1 is ubiquitously expressed (Gilbert et al., 2006; Iyer et al., 2003; Webber et al., 2008) whereas GRIF-1 and HAP1 displayed restricted expression and are enriched in neuronal cells (Beck et al., 2002; Kirk et al., 2006; Li et al., 1995). The HAPN domain family of proteins share an overlapping function in endocytic trafficking regulation but also have distinct roles in the regulation of other cellular processes. For instance, Trak1 and GRIF-1 also function in mitochondrial transport (Brickley and Stephenson, 2011; Fransson et al., 2006). In *Drosophila melanogaster* there is a single HAPN domain protein, Milton. Milton is an important regulator of mitochondrial transport; therefore, shares functional homology with Trak1 and GRIF-1. Studying Milton function in flies may represent a simpler model system to study the function of HAPN domain proteins, as there is only one. My finding that loss of Milton results in ectopic wing vein formation resembling EGF receptor/MAPK hyperactivation is the first to report that Milton may also share functional homology in the regulation of endocytic trafficking with the mammalian HAPN domain proteins. Thus, *Drosophila* is an excellent model to pursue further studies to reveal new genetic interactions that may have counterparts in humans. A conserved function between Milton and mammalian HAPN domain proteins would also make *Drosophila* an

excellent model to pursue the many questions that remain regarding the molecular machinery and biochemical mechanisms that regulate endocytic trafficking and mitochondrial dynamics.

Mitochondria are essential organelles that provide cellular energy, calcium buffering, and regulation of apoptotic cell death. The proper regulation of mitochondrial dynamics is critical to maintaining mitochondrial functions. The disruption of mitochondrial functions is increasingly implicated in neurological disorders and in a myriad of common disorders such as cancer, autoimmune disorders, and metabolic syndromes (Chen and Chan, 2009; Knott and Bossy-Wetzel, 2008; Lesnefsky et al., 2001; Pieczenik and Neustadt, 2007; Rehman et al., 2012; Wallace, 1999; Zuchner et al., 2004). Although the major protein regulators of mitochondria dynamics have been elucidated, their precise mechanisms of action remain unclear, and all the proteins that are involved are not known. Additionally, further research on the modulation of mitochondrial dynamics in the pathology of human diseases is needed.

Trak1 functions in mitochondrial transport and endosome-to-lysosome trafficking. For mitochondrial transport, Trak1 acts as an adaptor protein by directly binding the mitochondrial outer membrane Rho GTPase Miro and kinesin heavy chains to facilitate transport of mitochondria on microtubules. Because Trak1 is ubiquitously expressed and associated with several disease states I focused my studies on providing a better understanding of Trak1's role in mitochondrial dynamics. In my studies, I identified a novel function of Trak1 in mitochondrial fusion independent of its role in mitochondrial transport. Depletion of Trak1 results in mitochondrial fragmentation, which is linked to mitochondrial dysfunction and disease states. My findings indicate that Trak1 is an essential protein for maintaining normal mitochondria morphology, distribution, and cell survival under stress conditions. Understanding the role of Trak1 in regulating mitochondrial morphology and function will be instrumental in understanding how Trak1 normally protects against cellular dysfunction and disease. I found that the hyrt mutation of Trak1 linked to hypertonia (Trak1 hyrt) is partially mistargeted away from mitochondria to the cytosol. This result revealed a novel mechanism in hypertonia pathogenesis

in which the hyrt mutation may disrupt mitochondrial dynamics leading to impaired cellular health. Because mutations in Trak1 are directly linked to neurological disorders and cancer, a better understanding of Trak1 functions, both in mitochondrial dynamics and the endocytic pathway, will advance our understanding on the underlying disease mechanisms as well as provide possible therapeutic targets for these diseases.

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