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**UNIQUE AND SHARED FUNCTIONS OF KINESIN LIGHT CHAIN AND MILTON IN DROSOPHILA OOGENESIS AND AXONAL TRANSPORT**

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By
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ABSTRACT

Donna Lynn Fullerton

UNIQUE AND SHARED FUNCTIONS OF KINESIN LIGHT CHAIN AND MILTON IN DROSOPHILA OOGENESIS AND AXONAL TRANSPORT

The roles of Milton and Kinesin light chain (Klc) in ooplasmic streaming and axonal transport in live animals have not been studied. The heavy chain of Kinesin-1 (Khc) contains the ATP-dependent motor domain. Klc is a Khc associated protein long thought to mediate cargo linkage and shown to play a role in Kinesin-1 regulation. However, Milton can compete with Klc for binding to Khc and is thought to be the linker between Khc and mitochondria. To determine the roles of Milton and Klc *in vivo*, we used both zygotic mutants and RNAi to knockdown Klc and Milton in Drosophila oocytes and larvae and looked for changes in cytoplasmic streaming, *Oskar* mRNA localization and the axonal transport of both mitochondria and dense core vesicles (DCV). Knockdown of Klc disrupts *osk* mRNA localization and cytoplasmic streaming, but knockdown of Milton does not. Inhibition of Milton reduces the number of mitochondria in long axons and prevents most movement, but does not affect DCVs. Inhibition of Klc significantly reduces the transport of both mitochondria and DCVs. These data suggest that Klc is working as a critical factor in these Kinesin-1 driven transport events, including a previously undescribed role in mitochondrial transport.

## DEDICATION

I dedicate my dissertation work to the two loves of my life: my husband, Kurtis, and our daughter, Edith. I would not be the same person without you. You are my strength and my happy place. I love you and thank you.

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## CHAPTER 1

### Intracellular Transport During Drosophila Development

The components of cytoplasm are highly organized in cells (Porter *et al.*, 1945). This organization is important for cellular function and there are many ways that cells accomplish it (Mellman and Nelson, 2008). One important way that cells organize their cytoplasm utilizes adenosine triphosphate (ATP)-dependent motor proteins to move organelles and other macromolecular cargoes to the places that they are needed (Barlan *et al.*, 2013b). This sort of active intracellular transport is much faster than diffusion and especially important for cell function when the transport path is long (Vale, 2003). Humans with defects in intracellular transport can develop neurodegenerative diseases, such as Hereditary Spastic Paraplegia (Reid *et al.*, 2002), Parkinson's disease (Saha *et al.*, 2004), Charcot-Marie-Tooth (Goizet *et al.*, 2009; Crimella *et al.*, 2012), Perry syndrome (Farrer *et al.*, 2009), and Amyotrophic lateral sclerosis (De Vos *et al.*, 2007). Since intracellular transport is so important for life, further study of motor proteins is imperative.

### Kinesin-1

Kinesin-1, or conventional kinesin, was the first member of the kinesin motor family to be discovered and it will be the focus of this dissertation (Brady, 1985; Scholey *et al.*, 1985; Vale *et al.*, 1985). Kinesin-1 walks towards the plus ends of microtubules (Vale *et al.*, 1985) and often consists of a tetramer of two heavy chains (Khc) and two light chains (Klc) (Vale *et al.*, 1985; Bloom *et al.*, 1988; Kuznetsov *et al.*, 1988; Johnson *et al.*, 1990; DeBoer *et al.*, 2008). An N-terminal globular domain of Khc called the "head" or "motor domain" has ATPase and microtubule binding activity (Kuznetsov and Gelfand, 1986; Penningroth *et al.*, 1987; Bloom *et al.*, 1988; Hirokawa *et al.*, 1989; Scholey *et al.*, 1989). An alpha helical, coiled-coil forming stalk contains a binding site for Klc and an alternative light chain called Milton that serve as cargo linkers (Diefenbach *et al.*, 1998; Glater *et al.*, 2006). The stalk ends in a C-terminal globular tail that can inhibit the ATPase of the head (Seiler *et al.*, 2000;

Dietrich *et al.*, 2008; Moua *et al.*, 2011). While the motor domain provides the force for kinesin-1 driven transport, it is through the stalk domain and light chains that Khc attaches to the cargoes that it transports (Hirokawa *et al.*, 1989; Verhey and Hammond, 2009; Akhmanova and Hammer, 2010).

**Kinesin-1 Dependent Processes During *Drosophila* Development**

Many distinct kinesin-1 dependent transport processes have been discovered in Drosophila (Yang *et al.*, 1990; Martin *et al.*, 1999; Brendza *et al.*, 2000; Brendza *et al.*, 2002; Serbus *et al.*, 2005; Pilling *et al.*, 2006). These include the transport of mitochondria (Pilling *et al.*, 2006) and vesicles (Cai *et al.*, 2007) in axons, the posterior localization of *oskar* (*osk*) mRNA in mid-stage oocytes (Brendza *et al.*, 2000), and a mass cytoplasmic streaming that mixes late-stage ooplasm (Palacios and St Johnston, 2002; Serbus *et al.*, 2005). To gain new insight into how the Kinesin-1 complex is able to drive such diverse cargoes and processes, I have studied the contributions of Klc and Milton to them.

The axonal transport of mitochondria and vesicles is important for all eukaryotes, especially animals with neurons that have long axons. In the most extreme case, axons extending from the dorsal root ganglia of a blue whale can be up to 30 meters long (Smith, 2009). With most biosynthesis of new cytoplasmic components occurring in the neuron cell body the logistics of building and then maintaining a long axon present a serious challenge that must be met by vigorous long distance transport. The transport processes for mitochondria and vesicles have distinct delivery patterns, because mitochondria functions are required all along the axon (Hollenbeck and Saxton, 2005; Pilling *et al.*, 2006; Saxton and Hollenbeck, 2012) while vesicles carry membrane, membrane proteins, and neurotransmitters that are mainly needed in the distal axon at growth cones or later at synaptic terminals (Schwartz, 1979).

Some elements of axonal transport mechanisms are understood. One simplifying principle is that almost all axonal microtubule plus ends point toward the nerve terminal and minus ends point toward the neuron cell body (Burton and Paige, 1981; Heidemann *et al.*, 1981; Stone *et al.*, 2008; Zimyanin *et al.*, 2008). Since Kinesin-1 walks toward plus ends, it carries cargoes "anterograde" toward nerve terminals. A minus end motor, cytoplasmic dynein carries cargoes "retrograde" toward neuron cell bodies. Mitochondria and vesicle movement is saltatory, which means that individual organelles have frequent pauses and direction reversals, reflecting the interlaced functions of kinesins, dyneins, static anchorage systems, and regulatory mechanisms. Thus, although the transport path in axons is almost linear with highly ordered microtubules, the detailed mechanisms of axonal transport, including specific motor-cargo linkage schemes, are complex and not well understood.

Posterior concentration of *osk* mRNA in the developing oocyte is a crucial part of germline specification and posterior patterning (Ephrussi and Lehmann, 1992; Lasko, 1999; van Eeden and St Johnston, 1999; Brendza *et al.*, 2000). The mechanism for posterior localization of *osk* mRNA involves kinesin-1 driven saltatory movement of ribonucleoprotein (RNP) particles along microtubules. Oocytes are large (~300um diameter) and their microtubules are not as highly ordered as those of an axon. Minus ends are anchored to the cortex and plus ends are randomly distributed away from the cortex, with a slight bias of plus ends towards the posterior (Serbus *et al.*, 2005; Zimyanin *et al.*, 2008; Parton *et al.*, 2011). Current data suggest a mechanism in which saltatory *osk* RNPs, by following that slight bias, gradually concentrate at the posterior. There are many aspects of the mechanism of *osk* mRNA localization that remain to be elucidated. One important gap in our knowledge is how Kinesin-1 links to and thus moves *osk* RNP particles.

The third process I have studied is mass cytoplasmic streaming in late-stage oocytes. At the beginning of stage 10B, nurse cells that had been selectively transferring specific

mRNAs, proteins and organelles into the growing oocyte squeeze all of their remaining cytoplasm into the oocyte anterior. Since that cytoplasm is qualitatively different from the existing ooplasm, mixing is required to ensure normal development of the oocyte and future embryo. That mixing is accomplished by cytoplasmic streaming (Gutzeit and Koppa, 1982). A unique aspect of the ooplasmic streaming mechanism is that it is driven by a single motor type, Kinesin-1 (Palacios and St Johnston, 2002; Serbus *et al.*, 2005). Perhaps because it uses just kinesin-1, unopposed by dynein (Serbus *et al.*, 2005), and because there is no particular destination for the moving kinesin-cargo complexes, streaming is non-saltatory. The identity of the cargos that kinesin-1 carries to drive streaming is unknown. One important step toward a better understanding of the streaming mechanism would be to determine the influences of known kinesin-cargo linkers.

Overall, Kinesin-1 is essential for axonal vesicle and mitochondria transport, *osk* mRNA localization, and ooplasmic streaming during *Drosophila* development. While it is clear that Kinesin-1 is the driving force in these four processes, little is known about how it is adapted to solve such distinct transport objectives. To fill this gap, I have studied what is known about kinesin-1 linkage mechanisms and have tested the specific functions of different linkage factors.

**Kinesin-Cargo Linkage Mechanisms**

The functional diversity of Kinesin-1 is almost certainly facilitated by its different light chains and various associated proteins that control attachment to specific sets of cargoes. Studies have implicated Pat 1 (Loiseau *et al.*, 2010), Klc (Gindhart and Goldstein, 1996), SYD (Bowman *et al.*, 2000), Jip1/APLIP1 (Verhey *et al.*, 2001), APP (Kamal *et al.*, 2000), Ensconsin (Sung *et al.*, 2008; Barlan *et al.*, 2013a), Milton (Stowers *et al.*, 2002; Górska-Andrzejak *et al.*, 2003; Glater *et al.*, 2006) and Miro (Guo *et al.*, 2005; Saotome *et al.*, 2008; Wang and Schwarz, 2009) in Kinesin-1-cargo linkage. Some of these are also involved in

motor regulation. It has been hypothesized that careful regulation of Khc is necessary to prevent waste of cellular ATP, to prevent Khc from crowding microtubules, to prevent Khc from piling up at the plus ends of microtubules, and to control direction and stopping of motion.

*Klc*

Klc is a major player in Kinesin-1-cargo linkage and regulation (Stenoien and Brady, 1997; Gindhart *et al.*, 1998; Khodjakov *et al.*, 1998; Stowers *et al.*, 2002; Cox and Spradling, 2006; Glater *et al.*, 2006; Cai *et al.*, 2007; Wong and Rice, 2010). It is thought to bind cargos through its 6 protein-binding tetratricopeptide repeats (TPR) (Gindhart and Goldstein, 1996). Klc binds to a variety of proteins related to cargo attachment, including, Sunday Driver (SYD, JSAP, JIP-3) (Bowman *et al.*, 2000), APLIP-1/Jip-1 (Verhey *et al.*, 2001; Horiuchi *et al.*, 2005), and APP (Kamal *et al.*, 2000).

*Sunday Driver and Jip1/APLIP-1*

Sunday Driver (SYD, JSAP, JIP-3), was identified in a screen for proteins that cause axonal swellings like those caused by loss of Khc function (Hurd and Saxton, 1996; Bowman *et al.*, 2000). SYD is a c-Jun NH2-terminal kinase (JNK) scaffolding protein that contains a predicted transmembrane domain, and localizes to post-Golgi vesicles. SYD immunoprecipitates with Klc suggesting that Khc may link to some vesicles through Klc and SYD (Bowman *et al.*, 2000; Cavalli *et al.*, 2005)(Fig 1-1A).

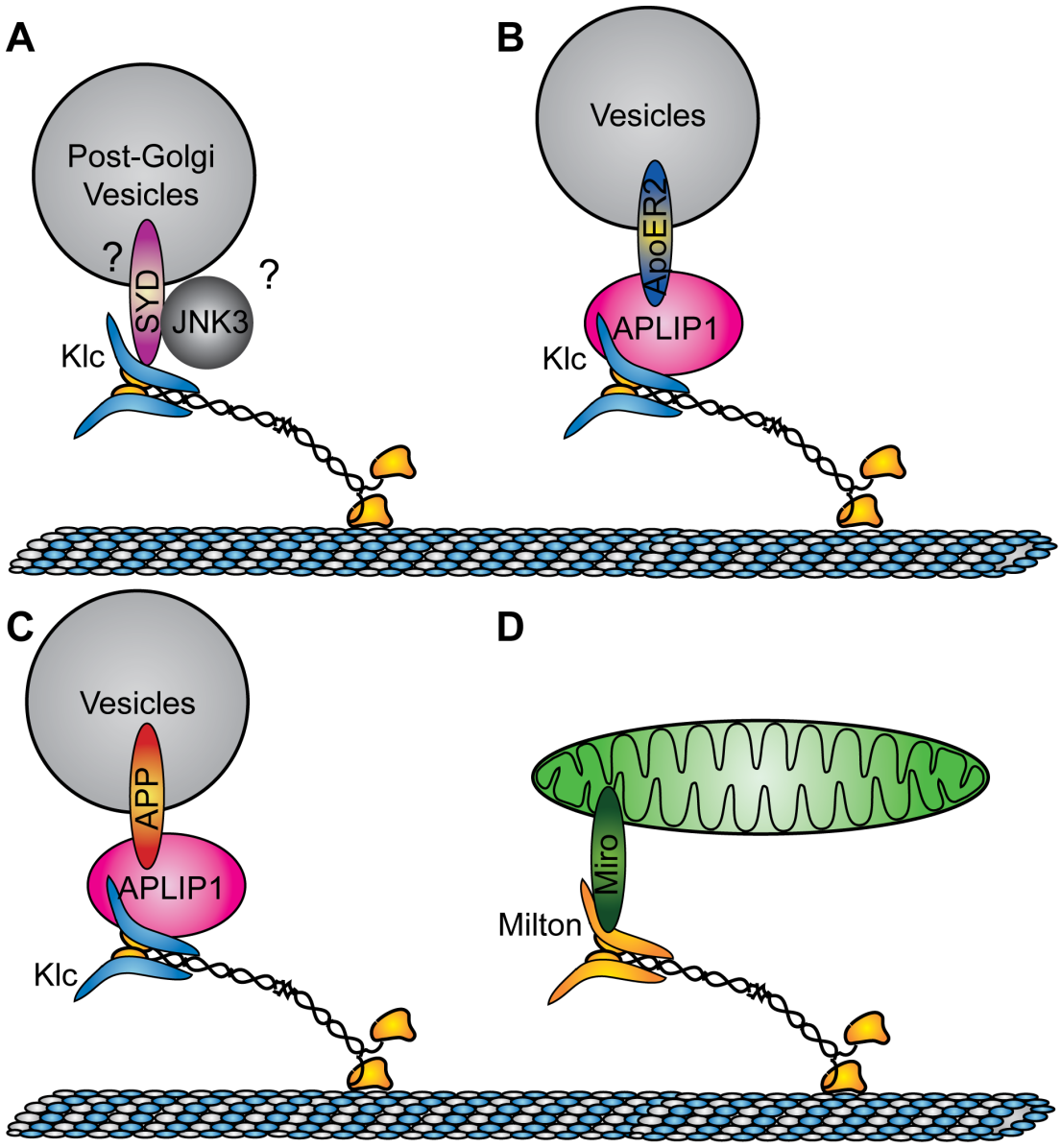



**Figure 1-1. Khc binds to cargoes via linker proteins.** (A) Khc (orange) binds to Klc (blue), which binds to SYD (purple), which has a putative transmembrane domain for binding vesicles and can bind to JNK3 (dark grey). SYD localizes to post-golgi vesicles. (B) Khc binds to Klc, which binds to APLIP-1 (pink), which binds to ApoER2 (dark blue), which has a transmembrane domain for embedding in vesicle membrane. (C) Khc binds to APLIP-1, which binds to APP (red), which binds vesicles through a transmembrane domain. (D) Khc binds to Milton (orange), which binds to Miro (dark green), which is present in the mitochondrial outer membrane.

APLIP-1, the Drosophila homolog of c-Jun N-terminal kinase (JNK) interacting protein 1 (JIP-1), which is another scaffold protein for the JNK pathway (Nihalani *et al.*, 2003), may be involved not only in attaching Khc to cargo, but also in regulating Khc (Verhey *et al.*, 2001; Horiuchi *et al.*, 2007). Jip-1 was shown to immunoprecipitate with Klc and ApoER2, a transmembrane receptor, and is thought to connect Khc to an unknown set of vesicles (Verhey *et al.*, 2001)(Fig 1-1B).

*APP*

Amyloid Precurser Protein (APP), a transmembrane protein that plays an important role in the pathology of Alzheimer's Disease (Selkoe, 2013), is suspected of functioning in Kinesin-1 cargo attachment (Kamal *et al.*, 2000; Kamal *et al.*, 2001). APP colocalizes with vesicle like structures, and axonal transport of vesicles carrying overexpressed APP depends on Kinesin-1 and Klc (Okada *et al.*, 1995; Kamal *et al.*, 2000; Kamal *et al.*, 2001). Together, these data suggest that APP links a species of vesicle to Khc through Klc (Fig 1-1C).

*Other Klc interacting proteins*

The TPR domains of Klc may also bind other proteins, including Kidins220/ARMS, Calsyntenin/Alcadein, collapsing response mediator protein-2, Huntington-associated protein-1, torsinA, 14-3-3, and Vaccinia virus's A36R protein (Stock *et al.*, 1999; Ichimura *et al.*, 2002; Kamm *et al.*, 2004; Ward and Moss, 2004; Kimura *et al.*, 2005; Konecna *et al.*, 2006; McGuire *et al.*, 2006; Araki *et al.*, 2007; Bracale *et al.*, 2007). These and other data suggest that diverse proteins can bind the TPR domains of Klc, and that those Klc interactors are important for determining the cargo-binding specificities of kinesin-1.

*Klc as a Regulator of Khc*

In addition to its role in cargo attachment, Klc has been shown to function in autoinhibition of kinesin-1 motor domain activity (Verhey and Hammond, 2009). Kinesin-1

can have two conformations, extended (~80nm long) and folded (~35nm long) (Hirokawa *et al.*, 1989). Studies using fluorescence resonance energy transfer (FRET) in transfected cells, deletion mutants in Neurospora, in vitro microtubule gliding assays, in vitro ATPase assays and sedimentation assays have shown that the folded form of Khc lacks ATPase activity (Hackney *et al.*, 1992; Verhey *et al.*, 1998; Coy *et al.*, 1999; Stock *et al.*, 1999; Seiler *et al.*, 2000; Cai *et al.*, 2007). It has been shown *in vitro* that the Khc tail inhibits the ATPase activity of its head (Coy *et al.*, 1999; Hackney *et al.*, 2009; Wong *et al.*, 2009). It is therefore thought that the folding of Khc allows the tail to interact with the head, thus inhibiting it, and that this inhibition of the head in the folded form is an important mechanism of regulation in the cell (Coy *et al.*, 1999). In the tail, a specific amino acid sequence, QIAKPIRP (The IAK domain), has been shown to interact with a part of the ATPase site in the head called Switch I and thus inhibit Khc ATPase and motor activity (Hackney and Stock, 2000; Seiler *et al.*, 2000; Cai *et al.*, 2007; Dietrich *et al.*, 2008).

It is not clear whether Klc activates Khc function *in vivo* or represses it (Cai *et al.*, 2007; Wong and Rice, 2010). A study using FRET in COS cells transfected with tagged rat Khc and Klc showed that in the presence of Klc, the Khc motor domains are pushed apart by a Klc subunit, which could inhibit processive stepping along microtubules (Cai *et al.*, 2007). Alternatively, an *in vitro* study using purified fragments of Drosophila Klc and Khc expressed in *E. coli*, showed that the addition of Klc reduced the affinity of the Khc head for the Khc tail and also reduced the affinity of the Khc head for microtubules (Wong and Rice, 2010). This supports a model in which Klc activates Khc, which seems to conflict with the data from Cai *et al* 2007. However, these two models may not be mutually exclusive, since other factors may be involved. For example, glycogen synthase kinase 3 (GSK3$\beta$) can phosphorylate Klc and thus decrease kinesin-1 association with membrane bounded organelles (Morfini *et al.*, 2002). This suggests that phosphorylation of Klc can inhibit Khc activity. Another study showed that binding of the protein Alcadein$\alpha$ (Alc$\alpha$) to a mouse Klc activated Khc binding to

vesicles and stimulated their anterograde transport. So it may be that Klc can either activate or inhibit Khc depending on post-translational modification and the association of other proteins (Kawano *et al.*, 2012). Perhaps post-translational modifications and associated proteins allow Klc to repress Khc during some processes and activate Khc during others.

It is evident that additional proteins can be involved in regulating Khc. The presence of inactive Kinesin-1 motors on purified membranes, suggests that binding to cargoes alone is not enough to activate the motor (Woźniak and Allan, 2006). It has been shown that Fez1 and Jip-1 are needed to release Khc from its autoinhibited state *in vitro* (Blasius *et al.*, 2007; Fu and Holzbaur, 2013). Another study suggests that activation of the MAPKKK or MAPKK components of the JNK pathway causes dissociation of kinesin-1 from the Jip-1 scaffolding protein (Horiuchi *et al.*, 2007). Additionally, neuronal injury that induces local activation of SYD (Jip-3, JSAP1) shifts anterograde transport to retrograde transport, and phosphorylation of Khc by JNK pathway proteins has been shown to negatively influence Khc binding to microtubules *in vitro* (Morfini *et al.*, 2006; Morfini *et al.*, 2009). These data support an important role for JNK proteins in activation of Khc (Cavalli *et al.*, 2005), and overall, it appears that many Klc associated factors can influence Kinesin-1 inhibition and activation.

*Ensconsin Activates Khc*

In addition to Klc, Fez 1, Jip-1 and other JNK signaling proteins, the microtubule associated protein, ensconsin, has been shown to increase Khc activity (Sung *et al.*, 2008; Barlan *et al.*, 2013a). In oocytes of *Drosophila ensconsin* mutants there is decreased localization of determinants, including *osk* mRNA (posterior), *staufen* (posterior), and *gurken* (anterodorsal) mRNAs (Sung *et al.*, 2008). Anterior *bicoid* mRNA localization was unaffected.

*Ensconsin* mutant oocytes have significantly reduced Khc-dependent cytoplasmic streaming. However, oocyte microtubule organization appears unaffected (Bulinski and Bossler, 1994; Sung *et al.*, 2008; Barlan *et al.*, 2013a). All of these in vivo effects are similar

to those caused by loss of Khc function (Brendza *et al.*, 2000; Brendza *et al.*, 2002; Palacios and St Johnston, 2002; Serbus *et al.*, 2005).

The Sung study used TIRF microscopy to test ensconsin influence on the movement of GFP-tagged *Drosophila* Khc on immobilized Alexa-568-labeled microtubules *in vitro*. While a truncated Khc, lacking the C-terminal half of the protein functioned the same in both wild type and ensconsin mutant ovary extracts, full length Khc showed a reduced velocity and displacement in ensconsin mutant ovary extracts. This suggests that ensconsin is required for Khc activity and that it regulates Khc through the motor's C-terminus.

A second study in *Drosophila* S2 cells and primary cell cultured neurons showed that ensconsin is necessary for Khc function (Barlan *et al.*, 2013a). S2 cells with induced neurite-like extensions, when treated with ensconsin siRNA showed decreased movement of mitochondria, peroxisomes, RNPs, lysosomes, and decreased microtubule sliding. Additionally, in primary cultured neurons from wild type and ensconsin mutant embryos, the distance traveled by mCherry labeled peroxisomes decreased in ensconsin mutants. Together, these data suggest that ensconsin is necessary for Khc dependent transport activity. The authors also generated a truncated human Khc construct with a mitochondrial targeting sequence. This recombinant Khc binds to mitochondria, but cannot autoinhibit. When this construct and a construct that cannot do autoinhibition folding were transfected into S2 cells that had thin neurite-like extensions, ensconsin RNAi had no effect. This is in contrast to wild type Khc, which shows decreased activity upon addition of ensconsin RNAi. These data suggest that ensconsin activates Khc by releasing it from autoinhibition.

*Pat1*

Klc was originally thought to link Khc to every cargo, but it is now known that there are other linker proteins, including Milton (Stowers *et al.*, 2002) and Protein interacting with

APP tail-1 (Pat1) (Loiseau *et al.*, 2010). Pat1 was first shown to associate with APP on vesicles (Zheng *et al.*, 1998). Later, Pat1 was shown to interact with Klc in a yeast two hybrid screen (Verhey *et al.*, 2001) and more recently it was shown to immunoprecipitate with Klc (Hammond *et al.*, 2008; Loiseau *et al.*, 2010). Knockdown of Pat1 disrupts posterior localization of the *osk* RNP particle protein Staufen in 22% of oocytes, but knockdown of both Pat1 and Klc disrupts localization in 78% of oocytes. The authors interpret this as likely redundancy in Pat1 and Klc function (Loiseau *et al.*, 2010), but it could mean that Klc and Pat1 are independently required for Staufen localization. Overall, these data suggest that Pat1 is involved in linking Khc to *osk* RNPs.

*Milton and Miro*

Milton was originally identified in a *Drosophila* screen for genes important for the function of photoreceptor neurons in adult eyes. Milton is necessary for mitochondrial transport and colocalizes with mitochondria (Stowers *et al.*, 2002; Górska-Andrzejak *et al.*, 2003; Cox and Spradling, 2006; Glater *et al.*, 2006). Milton competes for the Klc binding site on the Khc stalk (Glater *et al.*, 2006). Milton lacks any obvious sequence elements that would localize it to mitochondria. Therefore, the discovery of a mitochondrial membrane protein, mitochondrial Rho-GTPase (Miro), that interacts with Milton in a yeast two-hybrid screen and via immunoprecipitation was exciting and suggested a possible mechanism for Milton to link Khc to mitochondria (Giot *et al.*, 2003; Glater *et al.*, 2006). Mutations in the *Drosophila miro* gene cause axonal mitochondria transport defects (Guo *et al.*, 2005), which further supports a role for Miro in mitochondrial transport. Milton is not a well-conserved protein, so it isn't likely a universal adaptor between Kinesin and mitochondria, but Miro exists in all eukaryotes (Aspenström *et al.*, 2004; Frederick *et al.*, 2004; Fransson *et al.*, 2006). Since Miro is present on the mitochondria of many organisms, it is possible that Milton analogs connect Khc to Miro and mitochondria in other organisms (Fig 1-1D).

*Regulation of Khc by Calcium Through Miro*

Miro contains two $Ca^{2+}$ binding EF hands that are important for calcium dependent control of mitochondrial movement in cultured hippocampal neurons (Saotome *et al.*, 2008; Macaskill *et al.*, 2009; Wang and Schwarz, 2009). One study showed that a calcium-induced arrest of mitochondria transport was enhanced by the overexpression of Miro (Saotome *et al.*, 2008). Another study showed that calcium inhibits the transport of mitochondria through binding to the EF hands of Miro and that calcium allows Miro to bind and inactivate the Khc head (Wang and Schwarz, 2009). Together, these data suggest a model in which calcium binding to the EF hands of Miro allows it to interact with the Khc motor domain in a manner that inhibits motor-microtubule interaction. A study in cultured rat hippocampal neurons showed that increased calcium causes dissociation of kinesin-1 from the mitochondrial surface (Macaskill *et al.*, 2009). These two models for how calcium regulates Khc through binding to Miro are not mutually exclusive. What is clear from the data is that calcium can bind to Miro to inhibit Khc activity in mitochondria transport.

**Questions About Kinesin-1 Cargo Linkage Mechanisms**

In summary, the regulation of Khc function is essential in many organisms and is accomplished by many mechanisms and proteins, including Khc itself. The proteins that will be the focus of this dissertation, Milton and Klc, have roles in both physical attachment of Khc to cargoes and also in regulation of Khc. While ooplasmic streaming and *osk* mRNA localization are known to be Khc and microtubule dependent, the proteins and organelles involved in the transfer of force to the cytoplasm have yet to be fully explored and could involve either Klc or Milton. While it has been shown that Milton is important for mitochondrial transport and Klc is not (Glater *et al.*, 2006), no one has investigated whether Milton is involved in any other Kinesin-1 dependent processes. Additionally, the data in Khodjakov *et al* (1998) suggest that the simple model, where Klc and Milton have nonoverlapping roles, may not represent a universal cellular mechanism. While it is thought that Klc is involved in

linking Khc to vesicles, no one has looked *in vivo* at whether Klc is involved in vesicle transport. The question of which Kinesin-1 duties Milton and Klc are responsible for *in vivo* remains unanswered.

## CHAPTER 2

## The Role of Kinesin Light Chain in Ooplasmic Streaming, Oskar mRNA localization, DCV Transport and Mitochondrial Transport

### Introduction

The roles of Klc and Milton in early development and in axonal transport have yet to be fully explored. To study the role of Klc and Milton in early development and axonal transport, we used both zygotic mutants and RNAi to knock out Klc and Milton in living Drosophila oocytes and larva and used fluorescence in situ hybridization (FISH) to track *oskar* mRNA localization and time-lapse microscopy to observe the movement of yolk endosomes in streaming oocytes and two GFP tagged known cargoes of Kinesin in motor neurons. We also used immunostaining, using a new antibody against Drosphila Klc to see whether mitochondria are associated with Klc. Our results suggest that not only is Klc essential for osk mRNA localization and for transfer of force from Khc to the ooplasm during streaming, but also that Klc functions as a linker protein for mitochondria as well as vesicles.

## Materials and Methods

### *Genetics*

*Drosophila* were cultured using standard approaches (Barkus *et al.*, 2008). *Klc*$^{8ex94}$ and *Klc*$^{1}$ mutant strains were obtained from Joe Gindhart (Gindhart *et al.*, 1998). The maternal triple GAL4 driver (*MTD-GAL4*) which expresses GAL4 throughout oogenesis (Petrella *et al.*, 2007), the *OK6-GAL4* driver, which expresses GAL4 specifically in motor neurons (Sanyal, 2009), the *UAS-ANF-GFP* dense core vesicle marker (Rao *et al.*, 2001), and the mito-GFP mitochondria matrix marker (Pilling *et al.*, 2006) were obtained from the Bloomington Stock Center (Indiana University). The short hairpin RNAi strains *Klc-RNAi-1* (B#33934, y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00883}attP2), *Klc-RNAi-2* (B#36795, y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.GL00535}attP40 ), Klc-RNAi-3 (B#42597, y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS02429}attP40) and *Milton-RNAi* (B#43173, y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.GL01515}attP2) generated by the Transgenic RNAi Project (Harvard Medical School), and all other strains used were also obtained from the Bloomington Stock Center (Indiana University).

### *Axonal Transport*

To obtain zygotic *Klc* mutant larvae for axonal transport analysis *Klc*$^{1}$*/TM6B Tb* and Klc$^{8ex94}$/TM6B *Tb* adults carrying the OK6-GAL driver and either *ANF-GFP* or *mito-GFP* responders were mated and non-Tb progeny GFP-positive offspring were selected (Pilling *et al.*, 2006). Control larvae carried *OK6-GAL4* and the appropriate GFP responder over *CyO*. To obtain *Klc* or *Milton* RNAi larvae, adults homozygous for the appropriate RNAi transgene were mated with adults carrying *OK6-GAL4* and one of the GFP responders. Larvae that expressed GFP were cultured for 5-6 days. RNAi control larvae carried one copy of a non-specific short-hairpin RNAi (P{TRiP.HMS00883}attP2) with *OK6-GAL4* and one of the GFP responders.

To analyze axonal transport, third instar larvae were immobilized between two spaced coverslips in a sealed chamber using the human anesthetic desflurane (Baxter) as described previously (Djagaeva *et al.*, 2012). Segmental nerves were imaged through the ventral body wall using an Improvision Ultraview spinning disk confocal fluorescence system (BioRad) attached to a Nikon Eclipse TE2000-E microscope with a 60X 1.4NA objective. DCVs were imaged at 3.32 frames/s and mitochondria were imaged at 1 frame/s for 60-300s. Animals were released within 30min of anesthetization and data were used only if the larvae resumed normal activity. Image series were manipulated using ImageJ (imagej.nih.gov) and anterograde or retrograde flux was determined by counting the number of fluorescent objects/min crossing a line drawn perpendicular to a single nerve. Differences between mean flux values were assessed using a Student's t-test.

***oskar mRNA distribution***

$Klc^{8ex94}$ GLC were generated as previously described in Serbus, 2005 and Moua, 2011. Female progeny, *yw P{hs-FLP}/w; P{ovoD1-18}3L P{FRT($w^{hs}$)}2A/P{FRT($w^{hs}$)}2A $Klc^{8ex94}$,* were heat shocked at 30°C for 40 min two consecutive days. For *osk* fluorescent *in situ* hybridization, flies were dissected in Robb's medium (in under 3 minutes), then fixed, rinsed and probed as described previously (Cha *et al.*, 2002; Serbus *et al.*, 2005). Egg chambers were fixed and immunolabeled as described previously (Cha *et al.*, 2002; Serbus *et al.*, 2005). For immunolabeling, after egg chambers were dissected in PBS, egg chambers were fixed for 20 minutes in 600μl heptane mixed with a 200μl aliquot of fixation buffer (obtained from a tube containing 5μl NP-40, 100μl 20% EM grade paraformaldehyde, 900μl PBS). At the beginning of fixation, the sample was agitated vigorously by hand for 30 seconds and placed on a rocking platform for the remaining 19 minutes and 30 seconds. Ovaries were rinsed three times briefly in 0.2%Tween-20/PBS, then washed three times for 10 minutes in 0.2%Tween-20/PBS. The sample was blocked for 1 hour at room temperature

in 0.2%Tween-20/PBS containing 1% Bovine Serum Albumin, then resuspended in solution, containing rabbit anti-Klc 3196 affinity purified 1:1000 in 0.2% Tween-20/PBS. After primary antibody incubations went overnight at 4°C, the ovaries were rinsed three times, washed three times for 20 minutes per wash and resuspended in secondary antibody solution containing 1:500 donkey anti-rabbit Alexaflour 488 and 1:100 Texas Red phalloidin (Molecular Probes). After overnight incubation at 4°C, ovaries were rinsed three times, then washed three times for 20 minutes per wash, resuspended in 70% glycerol in PBS, mounted on slides, dissected gently to separate ovarioles, then sealed in with a coverslip secured around the edges with nail polish. Slides were imaged using a Leica SP5 Confocal Microscope. Imaged using a 250μm pinhole, a z-stack, using 1.5μm steps, at 1024 x 1024 pixel resolution.

***Ooplasmic streaming***

$Klc^{8ex94}$ GLCs were generated as described above. To make RNAi oocytes, P{otu-GAL4::VP16.R}1, $w^{*}$; P{w[+mC]=GAL4-nos.NGT}40; P{w[+mC]=GAL4::VP16-nos.UTR}$CG6325^{MVD1}$ (B# 31777) were mated to KlcRNAi-1, KlcRNAi-2, KlcRNAi-3, P{TriP.GL01515}attP2 (Milton RNAi, Valium 22, 3rd Chromosome, B#43173), KlcRNAi-2; Milton RNAi, KlcRNAi-3; Milton RNAi, P{UAS-LUC.VALIUM10}attP2 (Luciferase Control, B# 35788) or (8) $y^{1}$ $sc^{*}$ $v^{1}$; P{TRiP.GL00521}attP2/TM3,$Sb^{1}$ (Cherry red RNAi, B#36785). Ovaries were dissected in halocarbon oil 700 and transferred to a No. 1.5, 24 x 50mm glass coverslip (VWR). The ovaries were gently dissected to separate ovarioles. Time-lapse movies of cytoplasmic streaming in oocytes were taken as described previously (Danilchik and Denegre, 1991; Gutzeit and Arendt, 1994; Theurkauf, 1994; Theurkauf and Hazelrigg, 1998; Serbus *et al.*, 2005). Movies were recorded using a spinning disk confocal microscope (Nikon Eclipse TE2000-E) at 4 frames per minute for 3-5 minutes. 10 particles per movie were tracked using the Manual Tracking plugin

(http://rsbweb.nih.gov/ij/plugins/track/track.html) in ImageJ software (http://imagej.nih.gov/ij). The movies were divided into a grid and the particles to be tracked were chosen from a randomly selected grid section. The center of the endosome was selected with a cursor in each frame of the movie until the endosome disappeared. The distance between the initial and final position of the particle was divided by the time to calculate the velocity. An average velocity was calculated per oocyte and the Student's t-test was done to compare the averages of the wild-type and mutant oocytes. A p-value <0.05 was considered a significant difference.

***Immunostaining of oocytes and cultured neurons***

For immunolabeling of oocytes, after egg chambers were dissected in PBS, egg chambers were fixed for 20 minutes in 600μl heptane mixed with a 200μl aliquot of fixation buffer (obtained from a tube containing 5μl NP-40, 100μl 20% EM grade paraformaldehyde, 900μl PBS). At the beginning of fixation, the sample was agitated vigorously by hand for 30 seconds and placed on a rocking platform for the remaining 19 minutes and 30 seconds. Ovaries were rinsed three times briefly in 0.2%Tween-20/PBS, then washed three times for 10 minutes in 0.2%Tween-20/PBS. The sample was blocked for 1 hour at room temperature in 0.2%Tween-20/PBS containing 1% Bovine Serum Albumin, then resuspended in solution, containing rabbit anti-Klc 3196 affinity purified 1:1000 in 0.2% Tween-20/PBS. After primary antibody incubations went overnight at 4°C, the ovaries were rinsed three times, washed three times for 20 minutes per wash and resuspended in secondary antibody solution containing 1:500 donkey anti-rabbit Alexaflour 488 and 1:100 Texas Red phalloidin (Molecular Probes). After overnight incubation at 4°C, ovaries were rinsed three times, then washed three times for 20 minutes per wash, resusended in 70% glycerol in PBS, mounted on slides, dissected gently to separate ovarioles, then sealed in with a coverslip secured around the edges with nail polish. Slides were imaged using a Leica SP5 Confocal

Microscope. Imaged using a 250μm pinhole, a z-stack, using 1.5μm steps, at 1024 x 1024 pixel resolution, at 400 Hz.

To culture Drosophila neurons, flies expressing OK6-Gal4 UAS-mito-GFP were kept in apple juice vials with fresh yeast paste for 3 days. Embryos were collected after 24 hours. Embryos were washed in ethanol, then dechorionated in 50% bleach for 5 min, rinsed in E-wash (7% NaCl and 0.5% Triton X-100) and distilled water. 1 ml of Supplemented Schneider's medium (20% FBS, 200 ng/ml insulin, 500 U/ml penicillin and 50 μg/ml streptomycin in Schneinder's) was added and embryos were homogenized with a pestle. Lysate was centrifuges at 40 x g for 10 min.. Supernatant was transferred to a clean eppendorf tube and centrifuged at 380 x g for 10 min.. The supernatant was discarded and the cell pellet was resuspended in 200 μl Supplemented Schneider's medium and plated on Concavalin A-coated 25mm coverslips in 35mm petri dishes for 10 min to allow attachment before 2 ml Supplemented Schneider's medium with 20 μg/ml cytochalasin B was added to flood the plate. Cells were grown for 24 hrs in Supplemented Schneider's medium with cytochalasin B (cyt B). The media was changed after 24 hrs to remove cyt B and changed daily for 3-4 days to allow cells to grow neurites. Cells were immunostained as in Lu *et al* 2013 (Lu *et al.*, 2013) except cells were incubated overnight in primary antibodies; mαtubulin DM1α (1:1000), rαKLC (made by Strategic Diagnostics, Inc, 1:100) or rαKHC (Cytoskeleton, 1:100), chickenαGFP 1020 (Aves, 1:1000). Cells were incubated for 2 hours in secondary antibody; goat anti-chicken Alexa fluor 488 (life technologies, 1:1000), goat anti-mouse-Alexa fluor 568 (life technologies, 1:1000), goat anti rabbit- Alexa fluor 647 (life technologies, 1:1000). Cells were mounted in Prolong Gold mounting media (life technologies) overnight in dark at room temperature before imaging. Cells were imaged using the Nikon Structured Illumination Microscope (UCSF Nikon Imaging Center) with the Apo TIRF 100x Oil DIC N2 objective and an Andor DU-897 camera.

***Immunoblotting (for the eggs)***

Embryos laid by GLC females that had been mated to wild-type males were collected within 4hrs of egglay, frozen, then homogenized and subjected to SDS-PAGE and transferred to nitrocellulose. After blocking in Tris-buffered saline/Tween 20 with 5% nonfat dry milk, blots were incubated with a 1:500 dilution of anti-Khc (Cytoskeleton) and a 1:1000 dilution of rabbit anti-Klc made using a Klc peptide antigen by Strategic Diagnostics, Inc. and affinity purified using that same peptide. Blots were washed in TBST and then were incubated with the horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000) (Jackson ImmunoResearch Laboratories) diluted in blocking solution. Blots were again washed in TBST and secondary antibody localizations were detected using chemiluminescence (Advansta).

***RNA Quantification***

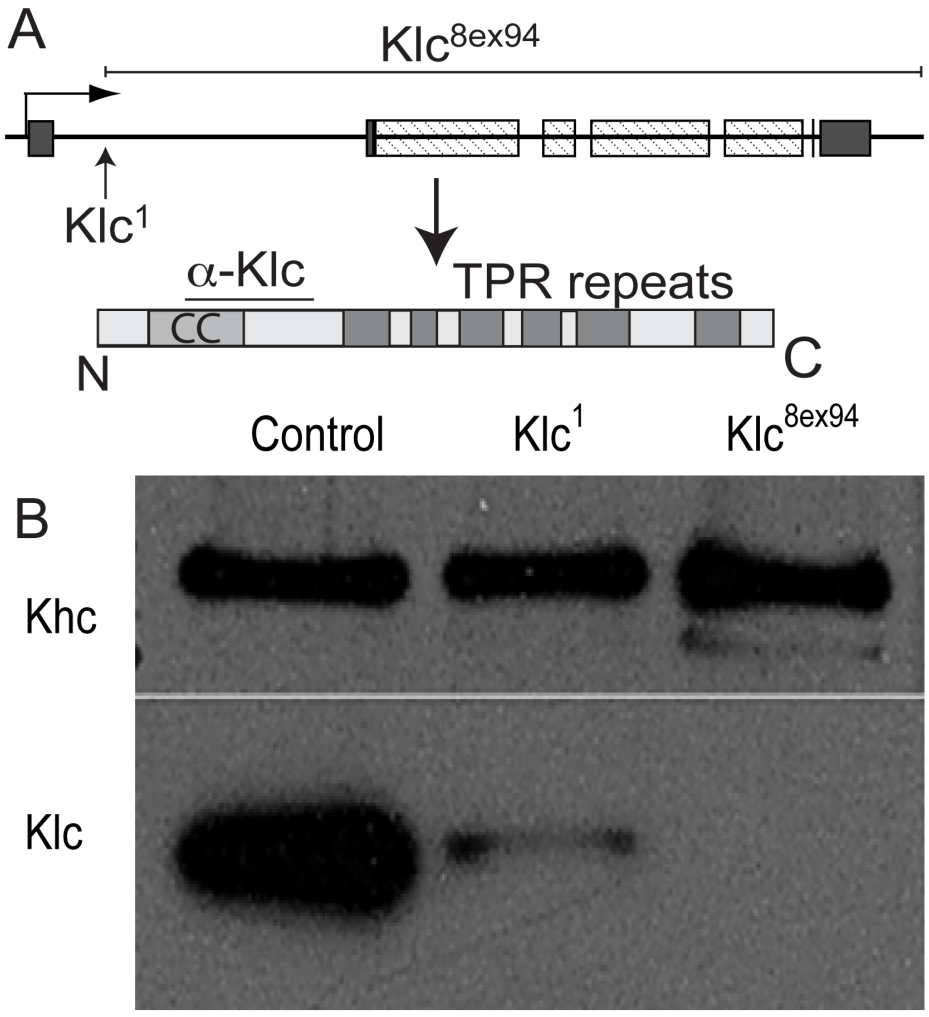
Total RNA was purified from 50 eggs collected from non-virgin females using RNeasy Mini Kit (QIAGEN) from the following genotypes: KlcRNAi-2 driven by Maternal Triple Driver B#31777 (MTD), KlcRNAi-3 driven by MTD, Milton RNAi B#43173 driven by MTD, KlcRNAi-2 (B#36795) and Milton RNAi both driven by MTD, KlcRNAi-3 and Milton RNAi both driven by MTD and mCherry B#35787 driven by MTD. Total RNA was purified from eggs using 〖PureLink〗^TM RNA Mini Kit (Ambion by Life Technologies) from the following genotypes: KlcRNAi-3 driven by MTD and mCherry B#35787 driven by MTD. cDNA was created from purified total RNA using SuperScript III in SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen by Life Technologies). cDNA was quantified through SYBR Green method using the LightCycler 480 (Roche). Klc (Left-ttgcttgcagaggggaacg Right-cgtactctgcacaacctggt), Milton(Left-ggagggtctgggcttgac Right-ggttgccgcatagaagga), and β-tubulin (Left-catccaagctggtcagtgc Right-gccatgctcatcggagat) primers were used separately to amplify target cDNA. The

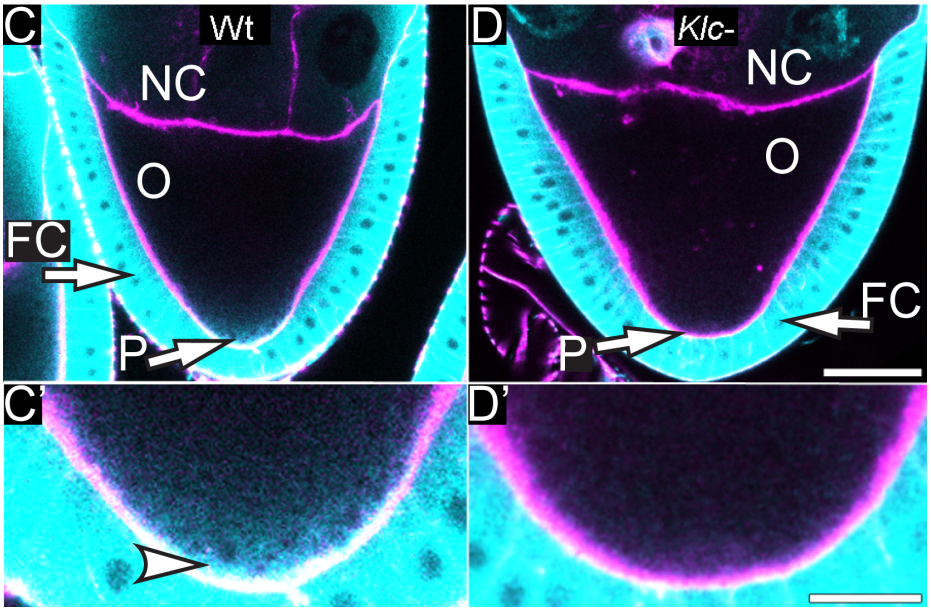
following equation was used to normalize Klc and Milton RNA signal to β-Tubulin RNA signal 2^(beta-tubulin - target sequence), which takes into account the exponential nature of PCR.

## Results

### *Klc is essential for early development*

To gain insight into the functions of Klc, we first studied its roles during early development by inhibiting its expression in the female germline. A mitotic recombination approach was used with two different *Klc* mutant alleles, $Klc^{1}$ and $Klc^{8ex94}$, to create homozygous mutant germline clones (GLCs) in otherwise heterozygous females. $Klc^{8ex94}$ deletes most of the *Klc* coding sequence (Fig 2-1A) and thus may cause a complete loss of function (Gindhart *et al.*, 1998). To check this and to assess perdurance of Klc from clone founder cells, freshly laid embryos produced from homozygous $Klc^{8ex94}$ GLC females that had been mated to wild-type males were subjected to western blot analysis with antibodies specific for a Klc peptide antigen (Fig 2-1A). Klc was detected in controls, at reduced levels in embryos from $Klc^{8ex94}$ GLCs, but none was detected in embryos from $Klc^{8ex94}$ GLCs (Fig 2-1B). Immunostaining of fixed egg chambers with the anti-Klc showed a concentration at the posterior pole in wild-type that was absent in $Klc^{8ex94}$ GLC oocytes (Fig 2-1C, D). These results show that $Klc^{1}$ causes reduced expression, that $Klc^{8ex94}$ is a null, and that Klc protein is not essential in the female germline for oocyte and egg production.


A
Klc8ex94
Klc1
α-Klc
TPR repeats
CC
N
C
Control
Klc1
Klc8ex94
B
Khc
Klc


C
Wt
NC
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D
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NC
O
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C'
D'

**Figure 2-1. Genetic elimination of maternal Klc.** (A, top) A diagram of the Klc gene is shown with the extent of the *Klc*$^{8ex94}$ deletion above and the location of the transposon insertion of *Klc*$^{1}$ below. Boxes show coding (striped) and non-coding (grey) exons with the transcriptional start site marked by a horizontal arrow. (A, bottom) The primary structure of Klc protein showing the location of the antigen used to generate and affinity purify Klc antibodies (a-Klc), the coiled-coil domain (CC), and tetratrico peptide repeats (TPR: dark grey boxes) (Gauger and Goldstein, 1993; Gindhart and Goldstein, 1996). (B) A western blot of eggs laid by *Klc*$^{8ex94}$ or *Klc*$^{1}$ GLC females probed with anti-Khc and anti-Klc antibodies. (C, D) Confocal fluorescence images of wild-type and *Klc*$^{8ex94}$ GLC egg chambers stained with anti-Klc (cyan) and with fluorescent phalliodin to show f-actin (magenta). High magnification images of the posterior regions (P) are shown in C', D'. Nurse cells (NC), follicle cells (FC), oocyte (O). The arrowhead in (C') indicates a posterior concentration of Klc staining that is absent in *Klc*$^{8ex94}$ GLC oocytes. Scale bars (C, D) = 40um, (C', D') = 15um. In the oocyte images, swap font sizes between the genotype labels and the structure labels. i.e. make the genotype more noticeable (and on a black rectangle) and the structure labels less prominent. Then remove the structure labels from the GLC panel because people should be able to understand based on the labels in panel C.

To determine if a lack of maternal Klc compromised later development, embryos from *Klc*$^{8ex94}$ GLC females were cultured. Out of hundreds observed, none hatched to become larvae. Since our GLC approach rendered most of the right arm of chromosome 2 homozygous, it is possible that unknown recessive mutations in essential genes other than *Klc* caused this maternal effect embryonic lethality. To test this, we inhibited *Klc* expression by crossing together a *UAS-Klc RNAi* transgene and a female germline-specific driver set, MTD-GAL4, that expresses GAL4 continuously during oogenesis (Petrella *et al.*, 2007). Quantification of *Klc* mRNA levels in 0-24hr embryos by qPCR indicated a 10-fold reduction (Fig 2-2). Consistent with the GLC results, after *Klc* RNAi females were mated to wild-type males, no embryos hatched into larvae. Thus, maternal Klc, like maternal Khc (Brendza *et al.*, 2000; Brendza *et al.*, 2002), is essential for early development.

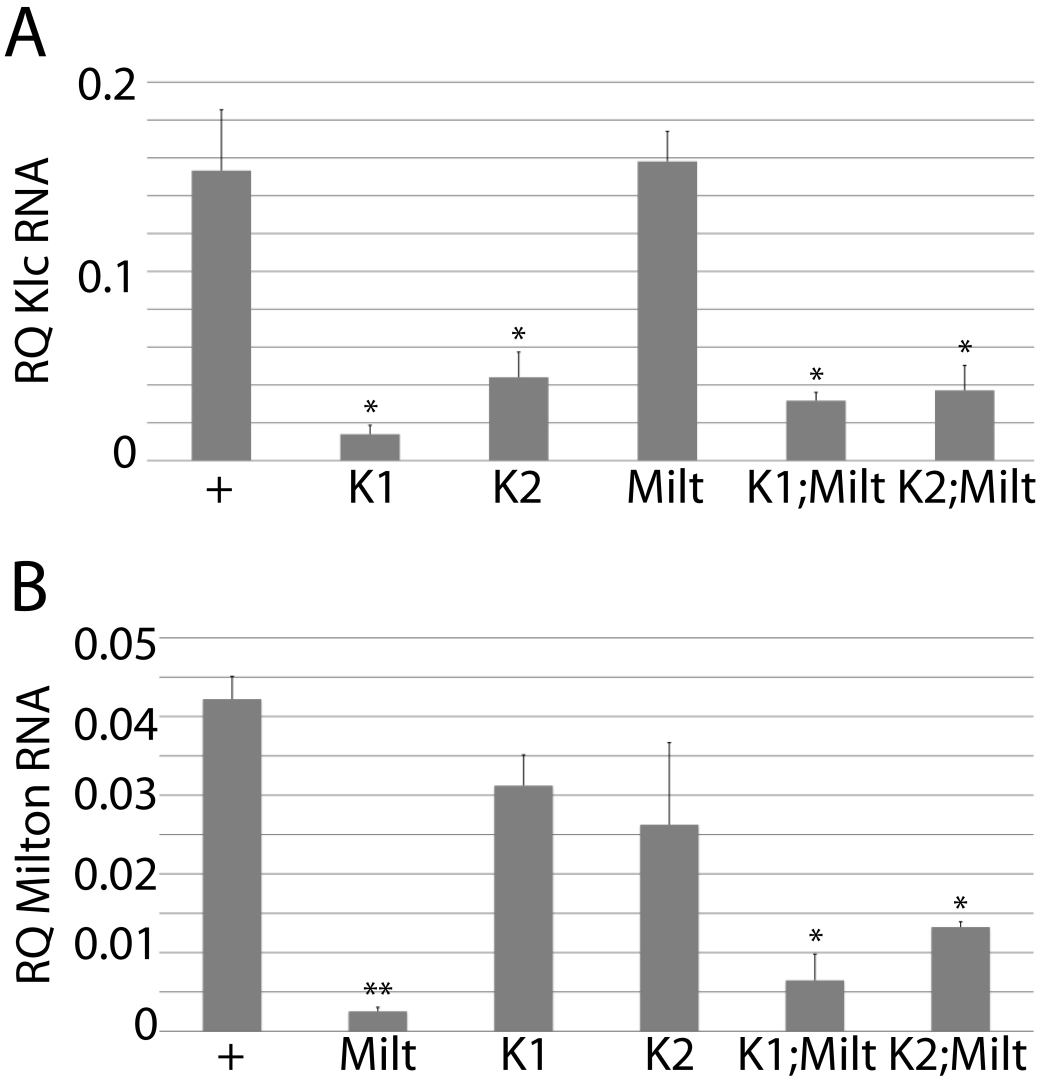


**Figure 2-2. Expression of Klc and Milton are knocked down in RNAi oocytes.** K1 and K2 are two different Klc RNAis. RQ is relative quantity of the indicated RNA compared to β-tubulin mRNA levels. (A) Expression of Klc normalized to β-tubulin expression. n=5 for the control, n=3 for K1, K2, Milt, K1;Milt and K2;Milt. (B) Expression of Milton normalized to β-tubulin expression. Error bars show standard error. n=3 for all genotypes. * shows a p-value<0.03. ** shows a p-value<0.002. K1 is B#42598. K2 is B#36795.

***Klc functions in oskar mRNA localization***

Kinesin-1 transports *oskar* mRNA in a random walk biased toward the oocyte posterior during stages 7-9, helping it concentrate there for anchorage to the posterior cortex (Brendza *et al.*, 2000; Serbus *et al.*, 2005; Zimyanin *et al.*, 2007; Parton *et al.*, 2011).

Absence of Khc function in *Khc* null GLC oocytes causes a failure of posterior *oskar* localization, leaving it distributed throughout the oocyte (Brendza *et al.*, 2000; Cha *et al.*, 2002; Zimyanin *et al.*, 2007). To test the influence of Klc on *oskar* mRNA localization, $Klc^{8ex94}$ GLC and *Klc* RNAi egg chambers were stained by fluorescence in situ hybridization (FISH) using a probe specific for *oskar*. In stage 9-11 $Klc^{8ex94}$ oocytes, *oskar* signal was abundant near the posterior, but often (74%, n= 31) was not focused into a normal cortical crescent (Fig 2-3). Substantial amounts appeared in irregular patches that trailed away toward the anterior. In some cases, small patches and diffuse signal were seen in central regions of oocytes (Fig 2-3B) These defective *oskar* localization effects are similar to those seen in oocytes from GLCs homozygous for partial loss of function *Khc* alleles (Serbus *et al.*, 2005; Zimyanin *et al.*, 2007; Moua *et al.*, 2011). Thus, while Klc makes significant contributions to localizing *oskar* mRNA at the posterior cortex, its role in that process is not synonymous with that of Khc. This is consistent with studies indicating that Klc is not an obligate component of the kinesin-1 motor complex (Palacios and St Johnston, 2002; Glater *et al.*, 2006; Rice and Gelfand, 2006; Loiseau *et al.*, 2010).

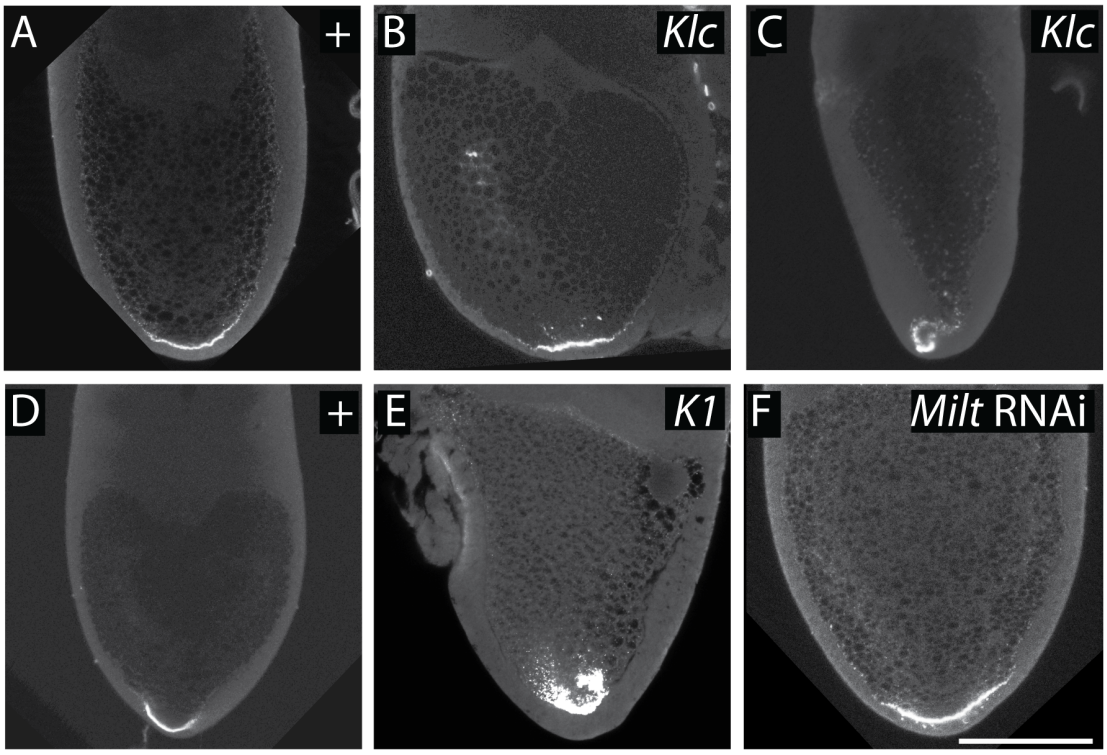


**Figure 2-3. Klc disruption hinders posterior *oskar* mRNA Localization.** Confocal micrographs show the distribution of a fluorescent oskar mRNA probe after in situ hybridization. The top row shows stage 10-11 oocytes that are A) wild-type GLC control (+) and B,C) $Klc^{8ex94}$ GLC null. The bottom row shows stage 10-11 oocytes that are luciferase RNAi control (+), E) *Klc*-RNAi-1 (K1) and F) Milton RNAi Note that in Klc GLC and RNAi oocytes, *osk* mRNA signal is present at or near the posterior, but distributions have a range of aberrations, including abnormal central spots (B), and contorted posterior localizations that are not tight against the oocyte boundary. Scale bar = 50um.

Kinesin-1 was discovered as a tetrameric complex with two copies of the force generator, Khc, and two copies of Klc, which is thought to function as a Khc regulator and cargo linker (Vale *et al.*, 1985; Akhmanova and Hammer, 2010). However, alternatives to Klc have been found. In metazoans, Pat1 and Milton can bind the Khc stalk and have other Klc-like functions (Zheng *et al.*, 1998; Loiseau *et al.*, 2010). *Pat1* null *Drosophila* mutants are viable and fertile, but 25% of their stage 9 oocytes show partial mislocalization of *oskar* mRNA complexes (Loiseau *et al.*, 2010). *Milton* null mutants are lethal in the second larval instar (Stowers *et al.*, 2002). *Milton* null GLC oocytes, can proceed through normal embryogenesis, but show aberrant transport of mitochondria into oocytes (Cox and Spradling,

2006). To determine if Milton influences *oskar* mRNA localization, a *UAS-Milton RNAi* transgene was expressed in the female germline using the MTD-GAL4 driver. Stage 9-11 oocytes (n = 81) had wild-type osk localization (Fig 2-3F), suggesting that Milton does not contribute to kinesin-1 driven oskar mRNA transport.

***Klc is a major contributor to fast ooplasmic streaming***

Fast ooplasmic streaming in *Drosophila* is a remarkable microtubule-based process that stirs oocyte cytoplasm with a massive influx of nurse cell cytoplasm (Gutzeit, 1986; Theurkauf *et al.*, 1992). At the start of stage 10B, concerted kinesin-1 motion along cortical microtubules both orders them into parallel arrays and drives coherent cytoplasmic flows along them (Palacios and St Johnston, 2002; Serbus *et al.*, 2005). Motor-fluid force transfer is mediated by viscous drag on kinesin-1 cargo complexes as they move away from cortically anchored minus-ends(Monteith *et al.*, *submitted* 2015). In *Khc* null oocytes there is no fast streaming (Palacios and St Johnston, 2002; Serbus *et al.*, 2005). To test the influence of Klc on fast streaming, live $Klc^{8ex94}$ GLC or *Klc* RNAi egg chambers were studied by time-lapse confocal fluorescence microscopy. In both cases, streaming was dramatically impaired, often appearing only in small areas, and suffering a 3-fold reduction in net velocity (Fig 2-4). This suggests that Klc links cargoes to Khc that contribute substantially to motor-fluid force transfer.

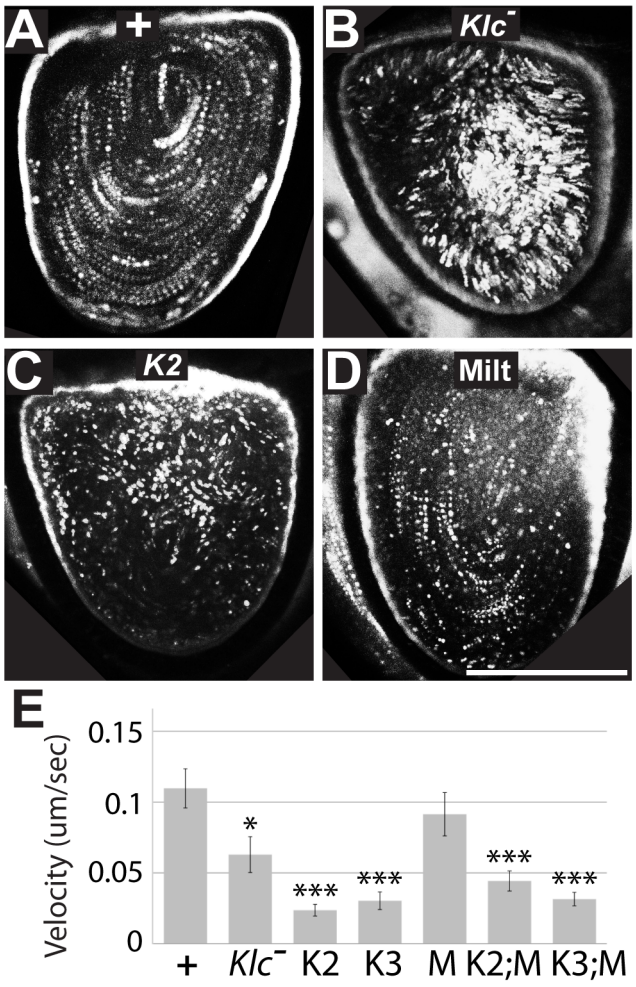


**Figure 2-4. Knockdown of Klc disrupts cytoplasmic streaming, but knockdown of Milton does not.** (A-D) show maximum projections of time-lapse image series of yolk endosomes during ooplasmic streaming. Dots reflect stationary yolk endosomes. Elongated dots and short streaks reflect yolk endosomes that moved slowly. Long streaks and lines of dots show yolk endosomes that moved fast for long distances. (A) RNAi control, n=7 (B) Milton RNAi, n=10 (C) Klc8ex94, n=8 (D) Klc-RNAi-2, n=10 (E) is a histogram of the average velocities of yolk endosomes from the above genotypes. +=luciferase control, $Klc^{-}$=$Klc^{8ex94}$, K2=Klc-RNAi-2, K3=Klc-RNAi-3, K2;M=Klc-RNAi-2;Milton double RNAi, K3;M=Klc-RNAi-3;Milton double RNAi, Error bars are standard error, * indicates a p-value<0.05. Scale bar 100um.

The residual streaming that occurs in oocytes without Klc could reflect the activity of Klc-independent cargo linkers. Pat1 is not a good candidate for this, because fast streaming patterns have been reported to be normal in *Pat1* null oocytes (Loiseau *et al.*, 2010). To address the possibility that Milton is responsible for the residual streaming, we tested it with *UAS-Milton RNAi* and the *MTD-GAL4* driver. Fast streaming velocity was not distinguishable

from wild-type (Fig 2-4E). Furthermore, streaming in *Milton-Klc* double RNAi oocytes not distinguishable from that observed with *Klc* RNAi alone (Fig 2-4E). These results indicate that there are Klc-independent regulation/linkage schemes that contribute to motor-fluid force transfer for fast streaming. An interesting alternative is that drag on kinesin-1 itself (without cargo) makes a significant contribution to motor-fluid force transfer (Monteith *et al.*, *submitted* 2015)

***Klc is required for the axonal transport of dense core vesicles.***

Neuropeptide-filled dense core vesicles (DCVs) formed at the Golgi are transported along microtubules to sites of secretion. Studies of *Drosophila* Khc missense mutations have shown that kinesin-1 inhibition causes focal accumulations along motor axons and severe reductions in the number of anterograde and retrograde moving DCVs (Djagaeva *et al.*, 2012; Lim and Saxton, *submtted* 2015). To determine if Klc contributes to DCV transport, a GFP tagged neuropeptide (ANF-GFP) that concentrates in DCVs, was expressed in motor neurons and imaged in abdominal segmental nerves of anesthetized larvae (Djagaeva *et al.*, 2012). To get third instar larvae that were well developed and suitable for axonal transport analysis, a hypomorphic *Klc* allele combination ($Klc^{1}/Klc^{8ex94}$) was used. ANF-GFP signal was prominent in focal accumulations along the lengths of nerves, and the number of moving DCVs (flux) was reduced ~10-fold in both anterograde and retrograde directions (Fig 2-5).

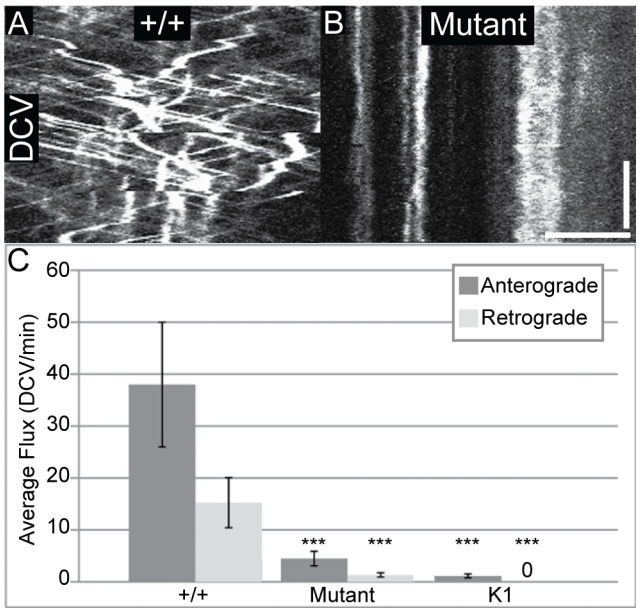


**Figure 2-5. Klc influences the axonal transport of dense core vesicles.** (A-B) Kymographs of time-lapse movies of transport in axons. A vertical line represents a stationary organelle, a line with a negative slope represents an organelle moving in the anterograde direction and a line with a positive slope represents an organelle moving in the retrograde direction. (A-B) are ANF-GFP tagged vesicles in larval axons A) kymograph of a control animal B) kymograph of a $Klc^{1}/Klc^{8ex94}$ mutant animal. Horizontal scale bars (B) are 5um. Vertical scale bars (B) are 10 seconds. C) Histogram of the flux of dense core vesicles. *** shows larvae that have a p-value ≤ 0.0007 using a Student's t-test. Error bars show standard error. Sample size is n= 10 larvae for all genotypes, except DCV transport in Klc-RNAi-1, where n=8 larvae.

Considering the possibility that pleiotropic effects of the *Klc* mutations in non-neuronal cells caused or contributed to those defects, a second approach was used. The effects of focusing Klc inhibition on neurons were observed by *D42-GAL4* driven expression of UAS-*Klc-RNAi*1 along with the *UAS-ANF-GFP*. Third instar larvae were robust, they exhibited distal paralysis (tail flipping), and they arrested in the pupal stage. Time-lapse microscopy of segmental nerves showed a 30-fold reduction in anterograde flux of DCVs and no retrograde flux (Fig 2-5C). This virtually complete inhibition of DCV motion suggests that all kinesin-1 mediated axonal DCV transport requires Klc.

***Klc is required for the axonal transport of mitochondria.***

Axonal mitochondria are transported anterograde by kinesin-1 (Pilling *et al.*, 2006). Because mitochondria are rare in *Milton* null axons, but are present in *Klc* null axons, and because of biochemical tests showing that Milton and Klc form separate complexes with Khc, it has been hypothesized that Milton is the Khc-mitochondria linker, and that mitochondria transport is independent of Klc (Glater *et al.*, 2006). To test the effects of Milton knockdown on transport, Milton mutant larvae expressing OK6-GAL4 and either UAS-mito-GFP or UAS-ANF-GFP. As expected from previous work (Glater *et al.*, 2006), we saw too few mitochondria in the axons to image. Conversely, we saw no effect on DCV transport (Fig 2-6). To test the effects of Klc inhibition on mitochondrial transport, *UAS-mito-GFP* driven in motor neurons by *OK6-GAL4* was imaged in larval segmental nerves (Fig 2-7). In $Klc^{1}/Klc^{8ex94}$ zygotic mutants, anterograde and retrograde flux were reduced 6-fold relative to wild-type. To test the possibility that this transport inhibition was due to pleiotropic physiological defects, Klc inhibition was focused specifically on motor neurons by *D42-GAL4* driven co-expression of *UAS-Klc-RNAi* and *UAS-mito-GFP*. Segmental nerves showed severely reduced mitochondria transport in both directions (>60-fold flux reduction). These results show that Klc has a major role in the axonal transport of mitochondria.

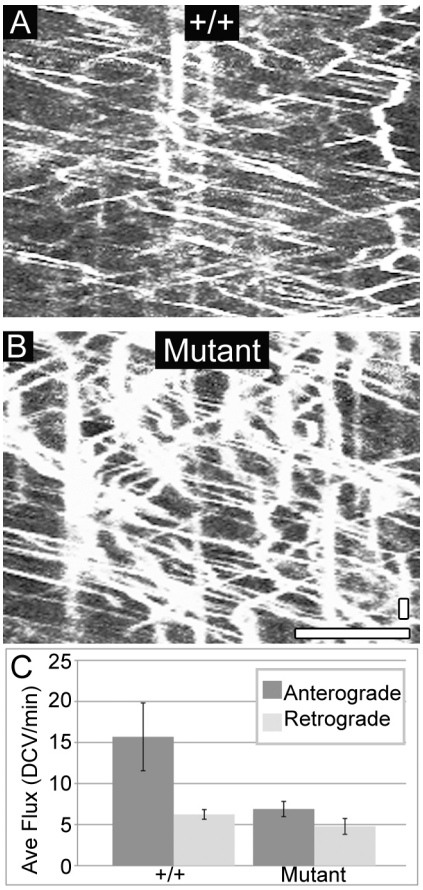


**Figure 2-6. Knockdown of Milton does not affect the transport of DCV.** (A & B) Kymographs of time-lapse movies of average flux of DCV in axons. A) kymograph of a control animal with UAS-ANF-GFP driven by D42-Gal4 B) kymograph of a Milton hypomorph/null (Milton$^{92}$/Milton$^{14514}$) animal with UAS-ANF-GFP driven by D42-Gal4. Horizontal scale bar (B) is 5um. Vertical scale bar (B) is 10 seconds. C) Histogram of the flux of dense core vesicles. The differences between wild type and mutant are not significant ($p>0.09$ for anterograde and $p>0.39$ for retrograde). Error bars show standard error. Sample size is n=5 larvae for both genotypes.

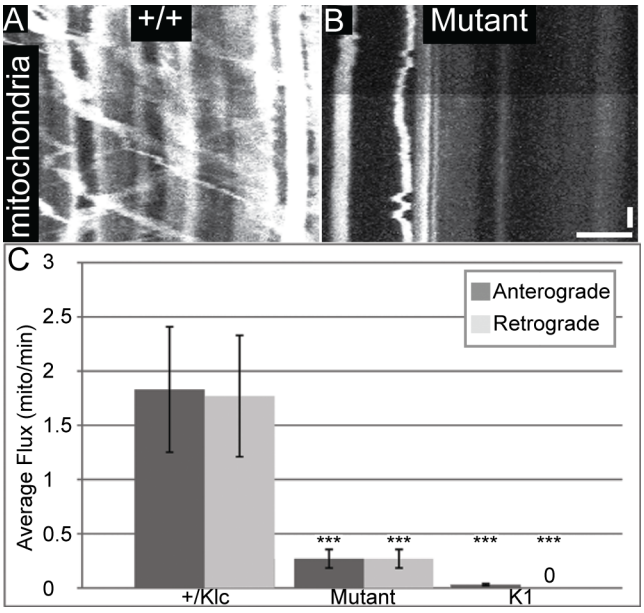


**Figure 2-7. Klc influences the axonal transport of mitochondria.** (A-B) Kymographs of time-lapse movies of transport in axons. A vertical line represents a stationary organelle, a line with a negative slope represents an organelle moving in the anterograde direction and a line with a positive slope represents an organelle moving in the retrograde direction. (A-B) are mitochondria tagged with GFP in larval axons A) kymograph of a control animal B) kymograph of a $Klc^{1}/Klc^{8ex94}$ mutant animal. Horizontal scale bars (B) are 5um. Vertical scale bars (B) are 10 seconds. B) Histogram of the flux of mitochondria. *** shows larvae that have a p-value ≤ 0.0007 using a Student's t-test. K1=Klc-RNAi-1. Error bars show standard error. Sample size is n= 10 larvae for all genotypes.

Since previous studies suggested that mitochondrial transport is independent of Klc (Glater *et al.*, 2006), it was surprising that knockdown of Klc caused a significant decrease in mitochondrial transport. This raised the question of whether the involvement of Klc in mitochondrial transport is as a linkage protein or regulatory protein. If Klc is involved physically with mitochondria, then it would be expected to be physically present on mitochondria. To test whether this is true, cultured neurons expressing mito-GFP were immunostained with affinity purified rαDKLC antibody and imaged using high-resolution microscopy (Fig 2-8). The data show that Klc is present both on mitochondria and elsewhere throughout the cell. There is likely cytoplasmic soluble Klc and Klc that is present on other organelles. The presence of Klc on mitochondria along with the data showing that knockdown

of Klc reduces mitochondria transport, supports a model in which Klc is physically involved in mitochondrial transport either as an attachment or regulatory protein.

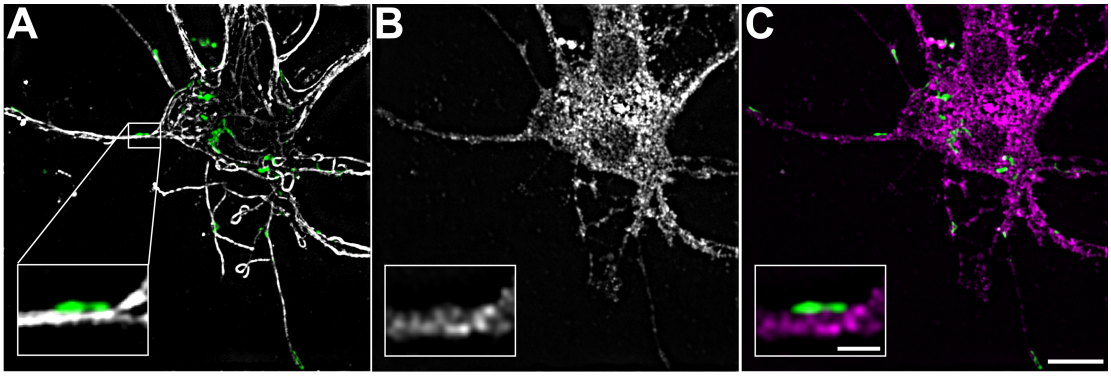


**Figure 2-8. Klc Colocalizes with Mitochondria in Cultured Neurites.** Cultured neurite made from cells expressing OK6mitoGFP. Panel (A) shows mitochondria-GFP in green immunostained with mαtubulin in white. (B) Immunostain of rαDKLC. (C) Composite with mito-GFP in green and rαDKLC in magenta. Scale bar is 5μm and 1μm in inset.

## CHAPTER 3

## Discussion

Using *in vivo* imaging methods and a new antibody to Drosophila Klc, we tested the influences of two Khc associated proteins, Klc and Milton, on *osk* mRNA localization, ooplasmic streaming and the transport of dense core vesicles and mitochondria to gain new insight into the mechanisms of kinesin-1 driven motility processes. We found that Klc is required for all four biological processes looked at, while Milton was only required for mitochondrial transport. This suggests that Milton is an attachment protein specific to mitochondria, while Klc helps Kinesin-1 to move multiple cargoes.

The lack of involvement of Milton in *osk* mRNA localization, ooplasmic streaming and DCV axonal transport agrees with previous research that Milton's role is specific to mitochondrial transport (Stowers *et al.*, 2002; Górska-Andrzejak *et al.*, 2003; Cox and Spradling, 2006; Glater *et al.*, 2006; Wang and Schwarz, 2009). The mechanism of ooplasmic streaming has yet to be elucidated, but evidence suggests that it is powered by Kinesin-1 moving along microtubules and it is hypothesized that it involves an "impeller" cargo that transfers the force produced by Kinesin-1 to the cytoplasm (Serbus *et al.*, 2005). The lack of Milton involvement in ooplasmic streaming suggests that mitochondria are not the impeller for streaming. Klc causes a dramatic reduction in streaming, thus the cargoes it links to Kinesin-1 may make strong contributions to force transfer. The residual streaming after Klc knockdon in oocytes suggests that naked Khc, with not attached cargos could generate enough viscous drag to drive streaming at reduced rates.

### *Oogenesis*

From these data, it is clear than Klc plays a role in *osk* mRNA transport and Milton does not. The nature of Klc's role is less clear. First, Klc could be part of the protein complex attaching *osk* mRNA to Khc. If Klc were acting as an attachment protein between Khc and the mRNA containing particle, then the *osk* localization phenotype of Klc knockdown might be

expected to be similar to that of a Khc knockdown, in which *osk* mRNA remains at the anterior of the oocyte (Brendza *et al.*, 2000). However, instead, we see a variety of patterns of *osk* mislocalization (Fig 2-3B-C & E-F).

Second, Klc could be acting as a regulator of Khc during *osk* mRNA transport. It is unclear whether Klc is acting as an inhibitor or activator of Khc activity *in vivo* (Cai *et al.*, 2007; Wong and Rice, 2010). Since *osk* mRNA is not getting to the posterior of Klc knockdown oocytes efficiently, the data suggest that if Klc is acting as a regulator during *osk* mRNA transport, then it is acting as an activator of Khc in this process.

Third, the affect of Klc knockdown on *osk* localization could be from indirect affects, such as reduced streaming velocity, disruption of microtubule organization or linking Khc to a protein required for *osk* mRNA localization other than *osk* itself. Since Klc knock down reduces the average velocity of fast ooplasmic streaming, it may also reduce the velocity of slow streaming. Similar *osk* mRNA spots and smears have been seen in at least three other studies (Palacios and St Johnston, 2002; Serbus *et al.*, 2005; Zimyanin *et al.*, 2007; Loiseau *et al.*, 2010). Central spots were seen in slow Khc mutants, Pat1 mutants and Pat1, Klc double mutants (Serbus *et al.*, 2005; Loiseau *et al.*, 2010). In Palacios *et al* 2002, the authors saw Stauffen localized to the posterior crescent 100% of the time with occasional dots near the posterior of the oocyte in Klc GLCs (Palacios and St Johnston, 2002). Since Stauffen tethers osk mRNA to the posterior pole, the presence of Stauffen in these dots suggests that *osk* mRNA may be present in them, too (St Johnston *et al.*, 1991). In Serbus *et al* (2005), the authors hypothesize that the aberrant central *osk* mRNA spots are due to slow transport and a two-step transport process. First, *osk* mRNA is transported by Kinesin-1 from the anterior to the center. Second, *osk* mRNA is transported by Kinesin-1 from the center to the posterior. They further hypothesize that in slow Khc mutants, *osk* mRNA takes longer to get to the center of the oocyte, which causes central spots to be seen in later stages of oogenesis. Then, the late central spots take longer to get to the posterior, which causes them to sometimes be visible in very late stage oocytes.

In Zimyanin *et al* (2007), aberrant *osk* mRNA spots were seen when *osk* mRNA was overexpressed. The authors found that in oocytes with these central spots, microtubule plus ends were localized both at the posterior and at the spots, in contrast to wild type oocytes in which microtubule plus ends are localized only at the posterior. The authors' speculate that the spot is caused by Oskar protein being translated before it reaches the posterior, which recruits plus ends of microtubules. This, in turn causes a positive feedback loop where more *osk* mRNA is transported to the center of the oocyte, which is translated into Osk protein, which recruits more microtubules. If the aberrant spots present in Klc knockdown oocytes are of a similar kind to the ones seen by Zimyanin *et al* (2007), then knock down of Klc could be causing *osk* mRNA to take longer to get to the posterior, which may initiate a positive feedback loop, which recruits both *osk* and microtubule plus ends to a central spot. Another study by the same lab showed that *osk* mRNA particles move with the slow ooplasmic streaming flows for part of the time and are pulled along microtubules part of the time (Zimyanin *et al.*, 2008). Since *osk* mRNA particles spend part of the time flowing with the cytoplasm in between bursts of plus end directed transport along microtubules, maybe slow streaming is important for increasing the chances of *osk* mRNA particles attaching to Khc and microtubules and loss of Klc could reduce that.

Another way that reduction of slow streaming velocity could cause aberrant *osk* mRNA localization is through the distribution of transcriptional repressors. It is thought that *osk* mRNA is transcriptionally repressed until it reaches the posterior by one of four proteins: Bruno, Apontic, p50 or Bicaudal-C (Rongo *et al.*, 1995; van Eeden and St Johnston, 1999). Maybe slow streaming is needed to disperse the protein that suppresses the translation of *osk* mRNA till it reaches the posterior. If this were true, then one might expect to see ectopic expression of Osk protein, which could initiate a positive feedback loop.

Another possibility is that Klc links Khc to another protein that is needed at the oocyte posterior for keeping *osk* mRNA there and instead of taking too long to get to the posterior, *osk* is getting there and falling off. This would explain the oocytes that look like the posterior

crescent is peeling off. Though it is not yet clear how knock down of Klc is disrupting *osk* mRNA localization, it is clear that Klc is important in this process.

These data have also shown that Klc plays an important role in ooplasmic streaming and that Milton does not. Previously published data showed that Klc is not necessary for ooplasmic streaming since some streaming occurs in Klc null GLCs, though the movements are less vigorous than wild type (Palacios and St Johnston, 2002). We have shown that the streaming that does occur in Klc knockdowns is significantly reduced (Fig 2-4). Again, since evidence suggests that Klc is a Kinesin-1-cargo linkage protein and a regulator of Kinesin-1, it is not simple to tease apart these two roles during cytoplasmic streaming. If Klc is working as a Kinesin-1-cargo linkage protein, then one might expect knockdown of Klc to show the same phenotype as a Khc knockdown, which slows the mean velocity of ooplasmic streaming to 30% of wild type and slows the peak velocity to about 5% of wild type (Serbus *et al.*, 2005). However, Klc knock downs behave more like slow Khc alleles, showing small pockets of streaming, but not organized or directed mass streaming (Serbus *et al.*, 2005). It is likely that Khc can bind to multiple cargos to drive streaming. One possibility, suggested by a collaboration between our lab and Josh Deutsch's lab, is that in the absence of an attached cargo, Khc alone could produce a small amount of streaming (Monteith *et al* 2015 *in prep,* (Deutsch *et al.*, 2011a; Deutsch *et al.*, 2011b). Additionally, in Khc null mutants, a small amount of indirect, disorganized movement still occurs. Some combination of these three possibilities may account for the 30% of wild type velocity remaining in Klc knock down oocytes.

***Axonal transport***

While the finding that Klc is necessary for DCV transport agrees with the thoughts in the field (Fig 3-1A), our finding that Klc is necessary for the transport of mitochondria is surprising based on previously published data (Stowers *et al.*, 2002; Glater *et al.*, 2006). Our evidence agrees with the finding of Glater *et al* (2006), which showed that there are still mitochondria

present in the axons of Klc knockout animals. However, the use of time-lapse imaging of mitochondria in live Drosophila larval neurons has allowed us to observe changes in the transport of mitochondria in Klc mutants that were beyond the capability of the methods used in Glater *et al* (2006).

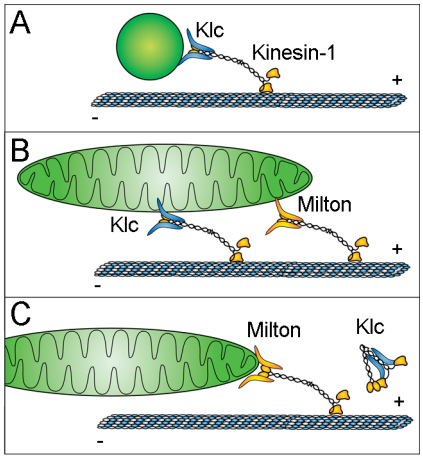


**Figure 3-1. A Role for Klc in Mitochondrial and DCV transport. (**A) represents a model in which Klc is the linker between DCV and Khc. (B) represents a model in which Klc and Milton are alternative linkers of Khc to Milton. (C) represents a model in which Klc is working as a regulator of mitochondrial transport, but not as a linker between Khc and mitochondria.

It has been shown via immunoprecipitation in mammalian cell culture that Klc and Milton do not bind to Khc at the same time and via immunostaining that mitochondria still travel into axons in Klc mutants (Glater *et al.*, 2006). It was inferred from these and other data that Klc is not needed for Khc to transport mitochondria. While this is likely true in their system, it is possible that an interaction between Drosophila proteins expressed in mammalian cells does not represent a true physiological interaction. Perhaps the tagged, transfected proteins were spliced differently or lacked some post-translational modifications or accessory proteins that are necessary for *in vivo* function. It has been shown that there is a specific isoform of Klc that is associated with mitochondria (Khodjakov *et al.*, 1998). In Drosophila, there are three isoforms but they have no changes in the coding sequence (Flybase.org). This suggests the possibility that Klc may play a role in the transport of mitochondria in a more physiologically relevant system.

There are a couple of possible explanations for our findings. One possibility is that mitochondrial transport in a Drosophila *in vivo* system works differently than in mammalian cell culture. Many of the proteins involved in these systems are highly conserved which suggests that the mechanisms across species are likely conserved. However, the conditions of cell culture may be nonphysiological enough to make the mechanism for the transport of mitochondria different than *in vivo*. It is possible that in live Drosophila larvae Klc can work as an alternative to Milton, also linking mitochondria to Khc or that both Klc and Milton are needed for mitochondrial transport (Fig 3-1B). Another possibility is that the role that Klc plays in mitochondrial transport has to do with its role in regulation of Khc autoinhibition (Fig 2-3C). Evidence seems to be conflicting and suggests that Klc may either be acting as positive regulator of Kinesin (Cyr *et al.*, 1991; Gauger and Goldstein, 1993; Stenoien and Brady, 1997; Wong and Rice, 2010) or a negative regulator (Hackney *et al.*, 1991; Matthies *et al.*, 1993; Jiang and Sheetz, 1995; Stock *et al.*, 1999; Cai *et al.*, 2007). The Cai *et al* (2007) study used FRET in mammalian cell culture to show that Klc pushes the Khc motor domains apart, possibly forcing it into an inhibited configuration. In contrast, the Wong and Rice (2010) study used tagged constructs of fragments of Drosophila Khc and Klc purified from *E. coli* to show that Klc reduces the affinity of the Khc head for the Khc tail and that Klc reduces the affinity of the Khc tail for microtubules, which suggests that it pushes Khc out of the autoinhibited configuration, activating it. The *in vivo* mechanism is likely more complicated than either of these models, but it is clear that Klc regulates Khc in some way, likely involving other proteins or post-translational modifications. If Klc inhibits Khc as the data in Cai *et al* (2007) suggests, then in Klc knockout animals Khc carrying mitochondria may not be stopping where the mitochondria are needed in the cell, thus mismanaging the energy requirements of the cell and making it sick. Mitochondria would be able to get out into the neuron, but the cell would be slowly dying, thus disrupting mitochondrial transport. On the other hand, if Klc activates Khc as the data in Wong and Rice (2010) suggests, then in Klc knockout animals Khc may simply be deactivated, reducing mitochondrial transport that way.

Another way that Klc may be affecting mitochondrial transport is by linking Khc to a cargo other than mitochondria that is important for mitochondrial transport or localization, such as a signaling protein or mRNA. The other cargo may be responsible for attachment of Khc to mitochondria or disrupting the association of Khc and mitochondria when they reach the place that they are needed in the cell. Mitochondria would be lacking some unknown Khc cargo that they need for their transport mechanism or to dock where they are needed. Yet another possible explanation is that Milton is required for transporting mitochondria into the axon, but Klc is required for transporting them once they are in the axon. While the current information cannot distinguish between the models further, it clearly supports a role for Klc in mitochondrial transport.

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