Axonal transport of mitochondria requires milton to recruit kinesin heavy chain and is light chain independent

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Introduction

Mitochondria are distributed within cells to match local energy demands. We report that the microtubule-dependent transport of mitochondria depends on the ability of milton to act as an adaptor protein that can recruit the heavy chain of conventional kinesin-1 (kinesin heavy chain [KHC]) to mitochondria. Biochemical and genetic evidence demonstrate that kinesin recruitment and mitochondrial transport are independent of kinesin light chain (KLC). KLC antagonizes milton’s association with KHC and is absent from milton–KHC complexes, and mitochondria are present in klc−/− photoreceptor axons. The recruitment of KHC to mitochondria is, in part, determined by the NH2 terminus–splicing variant of milton. A direct interaction occurs between milton and miro, which is a mitochondrial Rho-like GTPase, and this interaction can influence the recruitment of milton to mitochondria. Thus, milton and miro are likely to form an essential protein complex that links KHC to mitochondria for light chain–independent, anterograde transport of mitochondria.

Mitochondrial localization and transport ensure the proper inheritance of mitochondria upon cell division (Pereira et al., 1997; Yaffe, 1999) and position mitochondria where energy demands or oxygen supplies are greatest (Hollenbeck and Saxton, 2005). It is likely that the concentration of local cytoplasmic Ca2+ also depends on mitochondrial Ca2+ uptake (Werth and Thayer, 1994; Zucker, 1999). Consequently, mitochondria accumulate in subcellular regions with high metabolic requirements and high Ca2+ influx (Morris and Hollenbeck, 1993) and redistribute in response to changes in the local energy state (Wong-Riley and Welt, 1980; Hollenbeck, 1996). The transport of mitochondria is particularly vital in neurons because of their extended processes, and the disruption of mitochondrial transport is correlated with neurodegenerative disease (Hollenbeck and Saxton, 2005).

The mechanisms of mitochondrial transport differ between species and can require actin, microtubule attachment, or kinesins (Yaffe et al., 2003; Boldogh et al., 2005; Hollenbeck and Saxton, 2005). In metazoans, mitochondrial motility involves both actin- and microtubule-dependent mechanisms (Morris and Hollenbeck, 1995; Hollenbeck, 1996; Ligon and Steward, 2000b; Hollenbeck and Saxton, 2005). In particular, plus end–directed movement involves conventional kinesin (kinesin-1) motors (Hurd and Saxton, 1996; Tanaka et al., 1998; Pilling et al., 2006), although kinesin-3 motors are also implicated (Nangaku et al., 1994; Wozniak et al., 2005). Little is known about how mitochondrial kinesin is regulated or coupled to the organelle (Rintoul et al., 2003; Chada and Hollenbeck, 2004; Miller and Sheetz, 2004; Cai et al., 2005; Hollenbeck and Saxton, 2005; Malaiyandi et al., 2005; Minin et al., 2006).

We recently identified a novel protein called milton, which is required for mitochondrial transport within Drosophila melanogaster photoreceptors (Stowers et al., 2002). Mitochondria were absent from milton (milt) photoreceptor axons, but were normally distributed and appeared to be functional in their cell bodies. Although devoid of mitochondria, their axons and synapses were otherwise surprisingly normal in their general architecture, possessing microtubules, synaptic vesicles, and active zone specializations. Thus, the transport defect was selective for mitochondria (Stowers et al., 2002; Gorska-Andrzejak et al., 2003). The mechanism of milton’s action was unknown, but milton was associated with mitochondria and coimmunoprecipitated with kinesin heavy chain (KHC)
in extracts of fly heads (Stowers et al., 2002). The mammalian homologues milton 1 and 2, which are also called O-linked N-acetylglucosamine–interacting protein 106 (OIP106) and γ-aminobutyric acid A receptor–interacting factor-1 (GRIF-1), also colocalize with mitochondria and communoprecipitate with KIF5B, which is a mammalian homologue of D. melanogaster KHC (Beck et al., 2002; Iyer et al., 2003; Brickley et al., 2005; Gilbert et al., 2006). Therefore, we have suggested that milton acts as an adaptor or regulator of the mitochondrial anterograde motor.

We demonstrate a protein apparatus that recruits kinesin to mitochondria and thereby permits anterograde movement. Milton, which interacts with both KHC and the mitochondrial protein miro, is essential in this apparatus. In contrast, kinesin light chain (KLC) is dispensable for mitochondrial transport in axons.

**Results**

**Milton alters mitochondrial distribution**

Flies that are homozygous for milton die as second instar larvae, and milton transcripts are broadly expressed in these flies, suggesting a wider role for milton than its reported function in photoreceptors (Stowers et al., 2002). Therefore, we expressed GFP fused to a mitochondrial-import signal (mitoGFP; Pilling et al., 2006) in neurons of the central nervous system to examine mitochondrial distribution in first instar larvae that are homozygous for miltnull, which is a null allele. The segmental nerves that connect the central nervous system to the body wall of the larva contain motor and sensory axons, and thereby provide the clearest structures in which to image axonal mitochondria. In control larvae, numerous mitochondria were present in these axons. However, in miltnull larvae, axonal mitochondria were absent (Fig. 1, A and B). This defect is selective for mitochondria, as indicated by the continued presence of immunoreactivity for KHC, which is likely to transport many cargoes (Goldstein, 2001; Vale, 2003), and the synaptic vesicle marker synaptotagmin (Fig. 1, C–F).

We also assayed mitochondrial distribution in the ventral nerve cord, which consists of two central neuropil regions that run the length of the cord and are surrounded by a cortex of cell bodies (Fig. 1, G and H). The neuropil regions contain axons, dendrites, and pre- and postsynaptic endings. In control larvae, mitoGFP was present in the cell bodies, but was most abundant in the neuropil, reflecting the increased concentration of mitochondria at the synapses. In the miltnull mutant, the mitoGFP pattern was reversed, with little GFP remaining in the neuropil. Synaptotagmin localization was unchanged. Thus, the selective loss of mitochondria from axons and synapses is not restricted to photoreceptors. Moreover, milton probably also mediates mitochondrial transport in dendrites because at least half of the mitochondria of the neuropil are expected to derive from postsynaptic elements.

**Milton recruits KHC to mitochondria**

The mitochondrial transport defect in milton mutants, and the in vivo association between milton and KHC, suggests that milton is an adaptor that links KHC to mitochondria. To test this hypothesis, we transfected cDNAs encoding D. melanogaster milton (Stowers et al., 2002) and myc-tagged rat KIF5B (myc-KHC;
Verhey et al., 1998), either alone or together, into COS7 cells. *D. melanogaster* milton and its mammalian homologues function identically in all of our assays (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200601067/DC1; see Mammalian milton homologues); therefore, we have used the rat kinesin in these assays. Transfected alone, milton immunoreactivity was located exclusively on mitochondria (Fig. 2 B). In untransfected cells, the endogenous KHC was typically cytoplasmic, although in some cells KHC was also observed on mitochondria. Upon transfection with milton, the KHC became highly enriched on the mitochondria, with little remaining detectable elsewhere in the cell (Fig. 3 C). The phenomenon was more dramatic when rat myc-KHC was overexpressed in these cells; myc-KHC was largely cytoplasmic, and, in some highly expressing cells, colocalized with microtubules (Fig. 2 C), as previously observed (Verhey et al., 1998). However, when cells were cotransfected with both milton and myc-KHC, KHC was overwhelmingly located on mitochondria, colocalizing precisely with both MitoTracker and milton (64 out of 66 cells; Fig. 2, D1 and D2).

In addition, mitochondrial distribution was altered by the cotransfection of milton and KHC. When milton alone was highly expressed, the mitochondria were clustered near the nucleus in 90% of the transfected cells (Fig. 2 B), which is a phenomenon encountered in only 3% of control cells. At lower expression levels, the mitochondria remained distributed as in untransfected cells (not depicted). Overall, milton expression caused less clustering of mitochondria in COS7 cells than had previously been observed in human embryonic kidney 293T (HEK293T) cells, in which all of the mitochondria become localized in an aggregate near the microtubule-organizing center (Stowers et al., 2002). In contrast, coexpression of KHC and milton caused many, though not all, mitochondria to reside at the cell margin and to form clumps at the tips of cell processes. This redistribution was not caused by a general change in the cytoskeleton. Neither KHC nor milton, transfected singly or in combination, altered the arrangement of microtubules in these cells (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200601067/DC1). The microtubules are chiefly oriented with plus ends toward the periphery of the cell; thus, the

![Figure 2](http://www.jcb.org/cgi/content/full/jcb.200601067/DC1)

**Figure 2.** *Milton recruits KHC to mitochondria.* Transfected COS7 cells were immunostained with anti-milton mAb 5A124 (green) and anti-myc to detect myc-KHC (white). Mitochondria were labeled with MitoTracker orange (pink). The localization of the proteins was compared between sham-transfected cells (A) and those transfected with milton (B), myc-KHC (C), and milton and myc-KHC (D1 and D2). KHC is present in the cytosol and on microtubules in the absence of milton (B), but is recruited to mitochondria by the presence of milton (D1 and D2). Coexpression of these proteins caused mitochondria to form aggregates (arrows) that were frequently, though not always, at the periphery of the cell. Bar, 20 μm.
redistribution of mitochondria suggests that milton has recruited and activated coexpressed KHC and thereby caused a plus end-directed shift of many of the mitochondria. Together with the biochemical association of milton and KHC in *D. melanogaster* and the mitochondrial localization of milton (Stowers et al., 2002), the ability of milton to recruit KHC to mitochondria offers direct support for the hypothesis that milton is a mitochondria-specific adaptor protein for the kinesin-1 family.

**Mapping the requirements for milton–KHC interactions**

To further characterize the interaction between milton and myc-KHC, we mapped the region of milton that is required for their association by cotransfecting HEK293T cells and assaying the ability of portions of milton to coprecipitate with full-length myc-KHC. Milton comprises 1,116 amino acids with no recognizable structural motifs, except for a long, predicted coiled coil domain (residues 140–380) that contains a high degree of amino acid identity with the equivalent regions in mammalian milton homologues (Stowers et al., 2002). Milton 1–450 was sufficient to associate with myc-KHC. Milton 1–750 also coimmunoprecipitated with KHC, but the COOH-terminal domain of milton (Flag-tagged milton 750–1,116 and 847–1,116) did not (Fig. 4 A). We also mapped the interaction by looking for colocalization in transfected COS7 cells, whose larger cytoplasmic volume made colocalization easier to score than in HEK293T cells. Milton 1–450 colocalized with myc-KHC in aggregates, but these were not mitochondria. In contrast, Flag-milton 750–1,116 did localize to mitochondria, but in its presence myc-KHC remained cytoplasmic (Fig. 4 C). Thus, the associations of milton with KHC and mitochondria are separable, and the 1–450 region of milton is sufficient for the interaction with KHC.

We determined the region of KHC necessary for its association with milton by cotransfecting milton with each of three truncated myc-KHC constructs and then immunoprecipitating with anti-milton mAb 5A124. Milton associated with both full-length KHC and KHC lacking the last 64 amino acids of the tail domain (myc-KHC 1–891), but not with KHC lacking the entire tail domain (myc-KHC 1–810) or a larger deletion (myc-KHC 1–682; Fig. 4 B). Therefore, the KHC tail region 810–891, but not 892–955, was necessary for associating with milton. Consistent with this finding, we observed that myc-KHC 1–891, but not 1–682, could be recruited to mitochondria by milton overexpression (Fig. 4 D).

**Milton–KHC interaction is KLC independent**

The kinesin-1 family, including *D. melanogaster* KHC and mammalian KIF5s, are generally considered to be tetramers composed of two KHCs and two KLCs. The deletion of amino acids 810–891 of KHC diminishes KLC binding (Verhey et al., 1998), and because endogenous KLC was present in the aforementioned experiments, milton might interact either directly with KHC or via KLC. Therefore, rat HA-tagged KLC1 (isoform C; Verhey et al., 1998) was transfected into COS7 cells both alone and in combination with KHC and milton (Fig. 5). Alone, or when transfected with milton, KLC was cytoplasmic (Fig. 5 A; Verhey et al., 1998). Even when milton, myc-KHC, and HA-KLC were coexpressed, KLC was invariably cytoplasmic and not located on mitochondria (Fig. 5 D). Moreover, in these cells, myc-KHC was also always cytoplasmic (Fig. 5 E). Thus, KLC expression inhibited the recruitment of KHC to the mitochondria by milton. We demonstrated by coimmunoprecipitation that KLC similarly inhibited the interaction of milton and KHC; HA-KLC expression prevented the coprecipitation of myc-KHC and milton (Fig. 5, F and G). Although not significantly homologous, the KHC-binding regions of milton and KLC are both predicted coiled coils and, therefore, may have similar and competing interactions for a binding site on KHC.
KHC 1–891 is associated with mitochondria, which tend to be aggregated. The shorter KHC remains cytoplasmic in a milton-expressing cell, whereas (D) KHC 1–891, but not KHC 1–682, is recruited to mitochondria by milton. As only the former appears mitochondrial, whereas KHC remains cytoplasmic.

Figure 4. The interaction of milton and KHC. [A] Coimmunoprecipitation of truncated milton and myc-KHC from transfected HEK293T cells. [left] Cells were transfected with myc-tagged KHC and either full-length milton, milton 1–450, or milton 1–750. Total cell lysate was immunoprecipitated with anti-milton 2A108 (raised against residues 908–1,055) and then probed with SA124 and anti-myc (KHC). [B] Coimmunoprecipitation of truncated myc-KHC and milton from lysates of HEK293T cells. Cells were transfected with milton and myc-KHC deletion constructs as indicated and immunoprecipitated with anti-milton mAb 5A124 (raised against residues 908–1,055) and then probed with SA124 and anti-myc (KHC). [C] Coimmunoprecipitation of truncated myc-KHC and milton from lysates of HEK293T cells. Cells were transfected with milton and myc-KHC deletion constructs as indicated and immunoprecipitated with anti-milton mAb 5A124, and then the precipitate was probed with SA124 and anti-myc. (A and B) Expression of KHC in all of the lysates was confirmed by probing the original lysate [input] with anti-myc (KHC). In this and all subsequent figures, the autoradiograph exposures were chosen to show relative loading and protein expression levels, rather than to show the proportion of the protein in the lysate that was precipitated. [C] Milton 1–450, but not milton 750–1,116, colocalizes with KHC in COS7 cells. Anti-milton 2A108 (green) and anti-myc (KHC, white) label identical regions of cytoplasm (arrows) when KHC is expressed with milton 1–450, and these aggregates do not correspond to mitochondria labeled with MitoTracker orange (pink). Flag-tagged milton 750–1,116 (visualized with anti-milton mAb 5A124; green) and myc-KHC (white) do not colocalize, as only the former appears mitochondrial, whereas KHC remains cytoplasmic. [D] KHC 1–891, but not KHC 1–682, is recruited to mitochondria by milton. The shorter KHC remains cytoplasmic in a milton-expressing cell, whereas KHC 1–891 is associated with mitochondria, which tend to be aggregated at the margin of the cell [arrows]. Bars, 20 μm.

Milton splice variants are functionally distinct

Although, thus far, only a single milton cDNA, which is hereafter called milton-A, has been described (Stowers et al., 2002) and used in this paper, protein and RNA analysis suggested greater complexity. Using cDNAs and ESTs from the Berkeley Drosophila Genome Project, we found that milton splice variants can produce at least four distinct protein products with divergent NH2 termini. After differing 5′ ends, the transcripts converge in exon 9, at amino acid 129 of milton-A (Fig. 7 A). Anti-milton P1–152 antibody, which was raised against amino acids 1–152 of milton-A, recognized a single band on a Western blot of D. melanogaster extract, but anti-milton mAb 5A124, which was raised against a domain present in all the predicted splice variants (milton-A 908–1,055), recognized multiple bands (Stowers et al., 2002). These observations are consistent with expression of splice variants in vivo. The 129 amino acids of the NH2 terminus of milton-A are replaced by 136 amino acids in milton-B and 269 amino acids in...
These domains have little homology to either milton-A, or to one another. In milton-D, the NH2 terminus domain is replaced by an untranslated region, such that a translation start site corresponding to Met 138 of milton-A is predicted within exon 9. All of the predicted variants contain the predicted coiled coil domain. The National Center for Biotechnology Information databases of GenBank mRNAs and ESTs for human milton 1/OIP106 predict alternative NH2 termini that converge at the same point as the D. melanogaster variants, but no alternative NH2 termini are predicted for mammalian milton 2/GRIF-1 as yet.

Do all of the splice variants function equally in recruiting KHC to mitochondria? Each variant localized to mitochondria when transfected into COS7 cells (Fig. 7, A). They differed, however, in their interactions with KHC. Milton-D recruited myc-KHC to the mitochondria and coimmunoprecipitated with it (Fig. 7, B and H). Thus, milton’s KHC association domain is contained within the sequences that are shared by milton-A and the shorter milton-D. Together with our earlier data (Fig. 4 A), these results indicate that the KHC association domain resides within the region corresponding to 138–450 of milton-A. Because this sequence is also present in milton-B and -C, these isoforms were likewise expected to associate with KHC and recruit it to mitochondria when equivalent amounts were expressed. Surprisingly, this was only true for milton-B (Fig. 7, B and F); milton-C neither recruited cotransfected myc-KHC to mitochondria nor coimmunoprecipitated with another, KLC was associated with KHC, but not with milton. All samples were from one experiment and processed together, although they were run on more than one gel.

Figure 5. Mitochondrial transport by milton and KHC is independent of KLC. (A–E) COS7 cells, which were transfected as indicated, were immunostained with anti-milton mAb 5A124 (green) and anti-myc to detect myc-KHC (white), or with anti-HA to detect HA-KLC (white). Mitochondria were labeled with MitoTracker orange (pink). (A and B) KLC remains diffuse and cytosolic, whether or not milton is expressed. (C) In contrast, in the presence of milton, KHC was recruited to mitochondria, and, as shown in Fig. 2, mitochondria formed aggregates at the periphery of the cell (arrows). (D and E) When KLC and KHC are both expressed with milton, neither is concentrated on mitochondria, and the mitochondria remain dispersed. Bar, 20 μm. (F and G) Milton and myc-KHC do not coimmunoprecipitate in the presence of KLC. HEK293T cells were transfected with milton, myc-tagged KHC, and HA-KLC as indicated. The cell lysate was immunoprecipitated with anti-myc [KHC, F] or anti-milton mAb 5A124 [G], and immunoblots of both starting lysate (Input) and precipitates were probed with anti-myc, 5A124, and anti-HA. Milton and KHC no longer coprecipitate when KLC is coexpressed. (H) KLC is not part of the milton–KHC complex in vivo. Transgenic flies expressing myc-KLC were homogenized and immunoprecipitated with milton, anti-myc and anti-KHC. The starting homogenate and the precipitates (as labeled for each lane) were assayed as in F and G. Although milton and KHC coprecipitated with one another, KLC was associated with KHC, but not with milton. All samples were from one experiment and processed together, although they were run on more than one gel.
mitochondrial marker cytochrome granule neurons, endogenous milton colocalized with the reservation of the epitope (milton-A 1–152). In rat cerebellar which could recognize milton 1 and 2 because of the high con-

miltons in mammalian cells by means of the P1–152 antiserum,

mitochondrial transport.

mammalian homologues are likely to play equivalent roles in milton and its D. melanogaster chondria (Fig. S2 F). Thus, in COS7 cells, endogenous milton was also observed on mito-

nous for either klc92 or milt92. Third instar larvae were dissected and photoreceptor axons were labeled with anti-chaoptin (pink) and mitoGFP expressed selectively in the photoreceptors by ey-GAL4. The bundled photoreceptor axons defasciculated upon innervation of the optic lobe in both controls [A and C] and mutants [B and D]. [B] Although klc axons appear disordered, they still contain numerous mitochondria. [D] In contrast, milt axons project into the optic lobe more normally, but almost all of the axons lack mitochondria. A few axons do contain mitochondria, but they are likely to be heterozygous axons that persist at this developmental stage. Bars, 20 μm.

Therefore, we examined the distribution of endogenous miltons in mammalian cells by means of the P1–152 antisera, which could recognize milton 1 and 2 because of the high con-

servation of the epitope (milton-A 1–152). In rat cerebellar granule neurons, endogenous milton colocalized with the mitochondrial marker cytochrome c oxidase (Fig. S2 E). In COS7 cells, endogenous milton was also observed on mito-

chondria (Fig. S2 F). Thus, D. melanogaster milton and its mammalian homologues are likely to play equivalent roles in mitochondrial transport.

Miro binds to milton and can affect its association with mitochondria

Despite its mitochondrial localization, milton has neither a mitochondrial import sequence nor a transmembrane domain.

To examine how milton associates with mitochondria, we expressed partially deleted forms of milton. Milton’s COOH terminus (expressed as either amino acids 847–1,116 or 750–1,116) had a mitochondrial distribution in COS7 cells (Fig. 8, A and B), although some remained cytoplasmic in highly expressing cells. In contrast, milton 1–450 was primarily nuclear at low expres-

sion levels (Fig. 8 C) or filled the cytoplasm in higher expressing cells. Milton 1–750 was also cytoplasmic, although some remained cytoplasmic in highly expressing cells. In contrast, milton 1–450 was primarily nuclear at low expres-

sion levels (Fig. 8 C) or filled the cytoplasm in higher expressing cells. Milton 1–750 was also cytoplasmic, although some remained cytoplasmic in highly expressing cells.

Because miro has a transmembrane domain, we hypothe-

sized that miro might be important for the mitochondrial local-

ization of milton and that miro lacking the transmembrane domain (miro∆TM; amino acids 1–574) might have dominant-

negative effects. Unlike full-length miro, miro∆TM, when expressed in COS7 cells, was diffusely distributed throughout the cytoplasm and was also nuclear when very highly expressed. Moreover, miro∆TM did not alter mitochondrial distribution (Fig. 9 E). However, miro∆TM could still bind to milton, as indicated by their coprecipitation when cotransfected (Fig. 9 A). In contrast to the strictly mitochondrial localization of milton when expressed with full-length miro, milton was displaced from mitochondria in most cells by expression of miro∆TM (Fig. 9, F and G). Thus, miro appears to be important for the association of milton with mitochondria, perhaps by serving as a receptor for milton on the mitochondrial surface. Consistent with this hypothesis, a truncated milton (1–750), which did not associate with mitochondria when expressed alone (Fig. 9 H),
was recruited to mitochondria when *D. melanogaster* miro was overexpressed in COS7 cells (Fig. 9 I). Milton 1–750 was also able to coimmunoprecipitate with miroΔTM (Fig. 9 C). Notably this milton construct does not contain the mitochondrial association domain we identified in the COOH terminus; it is therefore likely that milton associates with mitochondria through at least two regions: residues 847–1,116 bind to an unidentified protein and 1–750 bind to miro (Fig. 10).

**Discussion**

We have examined the involvement of milton in kinesin-mediated mitochondrial motility and, thus, in the essential process of distributing mitochondria within the cell. From these studies we have derived a model of a protein complex that includes kinesin and adaptor proteins that link kinesin to the mitochondrion (Fig. 10). These proteins are also likely to serve as a focal point for regulating mitochondrial motility.

**The mechanistic basis of the milton phenotype**

In vivo, milton is required for the axonal transport of mitochondria throughout the nervous system (Fig. 1; Stowers et al., 2002). Milton associates with kinesin-1 via a highly conserved domain located between residues 138 and 450. This association can recruit kinesin to mitochondria in COS7 cells and appears to activate plus end–directed transport of mitochondria, as judged by their redistribution to aggregates in the periphery of many cells transfected with both milton and KHC. These findings provide a mechanistic explanation for the absence of mitochondria from milton axons and terminals. Consistent with this model, the motors that endogenously associate with milton, KHC in *D. melanogaster* (Fig. 5 H; Stowers and Schwarz, 2002), and KIF5 members in mammals (Fig. 5, F and G; Brickley et al., 2005) have previously been implicated in the axonal transport of mitochondria (Hurd and Saxton, 1996; Tanaka et al., 1998).

**The function of miro in mitochondrial motility**

The association of milton with mitochondria appears to be mediated, in part, by its interactions with miro, and this probably accounts for the failure of mitochondrial transport in the axons of *miro* mutants (Guo et al., 2005). This proposal is supported (a) by the ability of a truncated cytosolic form of miro to act as a dominant negative and displace milton from mitochondria, and (b) by the ability of overexpressed full-length miro to recruit to mitochondria a truncated milton (residues 1–750) that could not independently localize there. However, additional interactions for tethering milton to mitochondria are likely, as a COOH-terminal portion of milton (residues 847–1,116) also localizes to the organelle. The difficulty of purifying mitochondria from limited numbers of homozygous *miro* larvae prevents a direct determination of the amount of milton on mitochondria that lack miro.

The role of *miro* in kinesin-mediated transport does not preclude additional roles for *miro*. Indeed, such functions are
likely because a miro homologue, GEM1p, is found in yeast, where mitochondrial motility is chiefly actin-based (Yaffe, 1999; Boldogh et al., 2005), and GEM1 mutants have abnormal mitochondrial distributions (Frederick et al., 2004). In addition, it will be of interest to determine the relationship of milton and miro to syntabulin, which is another protein that has recently been proposed to link kinesin to mitochondria (Cai et al., 2005).

**KLC is not needed for KHC to transport mitochondria**

Unexpectedly, we found that axonal transport of mitochondria did not require the light chains of the kinesin-1 motors and that light chains were, indeed, absent from the milton–kinesin complex. When expressed in COS7 and HEK293T cells, the association between milton and KHC was inhibited by KLC. In fly homogenates, KLC was not detected in immunoprecipitates of the milton–KHC complex. Mitochondria were abundant in the axons of klc−/−photoreceptors. Thus, this mitochondrial motor provides an exception to the conventional tetrameric structure of kinesin-1. Precedent for KHC-based transport that is KLC independent has been reported in Neurospora crassa (Steinberg and Schliwa, 1995), sea urchins (Skoufas et al., 1994), neuronal dendrites (Setou et al., 2002), and the transport of RNA particles (Palacios and St. Johnston, 2002; Kanai et al., 2004; Ling et al., 2004).

The interaction of milton with KHC was not only KLC independent, but was inhibited by KLC overexpression in transfected COS7 and HEK293T cells. Therefore, a pool of KHC without KLC is required for milton to associate with KHC in vivo. Previous studies have found evidence for such a pool in bovine brain (Hackney et al., 1991). In light of our findings and those cited in the preceding paragraph, it may be appropriate to consider the light chains as one of several cargo adaptors for kinesin-1, of which milton is another.

**The regulation of mitochondrial movement**

Mitochondria are not static. In dividing cells they go through orchestrated movements to distribute themselves between the daughter cells (Yaffe, 1999). Within axons they typically alternate between stationary and moving states and can reverse their direction (Hollenbeck, 1996; Ligon and Steward, 2000a). They arrest in the presence of elevated Ca2+, including Ca2+ that is derived from the activation of synaptic receptors (Li et al., 2004), and respond to the activation of neurotrophin receptors and various intracellular signals (Rintoul et al., 2003; Chada and Hollenbeck, 2004; Miller and Sheetz, 2004; Malaiyandi et al., 2005). It is noteworthy that, in addition to linking kinesin to the mitochondria, the milton–miro complex provides several possible mechanisms for the regulation of transport. These include the alternative splicing of milton, the posttranslational modification of milton, and the modulation of the state of miro.

We have shown that the choice of NH2 terminus splicing variant can influence KHC’s association with the adjacent region of milton. In particular, KHC did not associate with milton-C, although it contains the KHC-association domain that is common to all the isoforms. The NH2 terminus of milton-C presumably inhibits the interaction with KHC and might, thereby, reserve a pool of mitochondria for retention in the cell body. Alternatively, the inhibition may not be constitutive in vivo, but, instead, might undergo regulation by additional factors and thereby control the recruitment of kinesin. In this context, it may be noteworthy that multiple bands of milton are detected on immunoblots from fly heads. Most of the milton isoforms in these homogenates are in an association with KHC, as determined by immunodepletion with anti-KHC. However, there is one major band, representing nearly half of the endogenous milton, which does not appear to be associated with KHC (Stowers et al., 2002). Thus, additional motors may associate with milton, and particularly with milton-C. Milton may also be
involved in such processes as mitochondrial fission and elongation, and such a role might explain the clustering of mitochondria when milton and miro are overexpressed.

The alternative splicing of milton may also represent an adaptation of the complex to the needs of particular cell types. Antiserum P1–152, which binds only to milton-A, labels a subset of the structures in the D. melanogaster brain that are recognized by antibodies to the common regions (Stowers et al., 2002). Thus, there is tissue specificity in the choice of splicing variant. To date, ESTs for milton-D have only been found in a testes library; therefore, milton-D may correspond to the male-specific milton transcripts on Northern blots (Stowers et al., 2002) and be necessary for the elongation of mitochondria along the axoneme of sperm (Siegenthaler et al., 2003).

Posttranslational modifications are also likely to regulate mitochondrial motility. In particular, the COOH-terminal portions of the mammalian miltons bind to, and are substrates for, the cytosolic glycosylating enzyme O-GlcNAc transferase (OGT; Iyer et al., 2003). We have identified D. melanogaster OGT by mass spectroscopy in immunoprecipitates of milton.

Figure 9. Milton associates with miro, and a dominant-negative miro can displace milton from mitochondria. (A–C) Coprecipitation of milton and T7-tagged miro constructs from transfected HEK293T cells with antibodies to T7. (A) Milton copurified with full-length miro, as well as miro lacking the COOH-terminal transmembrane domain (Miro ∆TM). (B) Both milton-A and -D coprecipitated with miro. (C) Milton 1–750 communoprecipitates with miro ∆TM. (D and E) Full-length miro localizes to mitochondria when transfected into COS7 cells (D), but miro ∆TM does not (E). (F–I) Cotransfection of full-length and truncated milton and miro constructs. Milton and miro colocalize with mitochondria when simultaneously transfected (F), but milton is displaced from mitochondria when cotransfected with miro ∆TM (G). Milton 1–750 is not mitochondrionally associated when expressed alone (H), however, coexpression with miro induces mitochondrial localization (I). Bar, 10 μm.
Mitochondria were visualized in D. melanogaster using transgenic stocks containing mitoGFP (Pilling et al., 2006) and placed under the control of a UAS promoter. Expression in selective tissues was driven by D42-Gal4, which is expressed in a subset of neurons (Fig. 1), or ey-Gal4, which is expressed in photoreceptors (Fig. 6). To visualize mitochondria in culture, cells were incubated with 100–300 nM MitoTracker orange (Invitrogen) for 15 min.

**Constructs**

Milton-A deletion constructs were constructed as follows: milton-A 1–450 and milton-A 1–750 were made from full-length milton-A in pCMV Tag1 (Stowers et al., 2002) that was partially digested by SalI; milton-A 608–1,116, 750–1,116, 608–942, and 847–1,116 were made by PCR with 5′ primers containing a BamHI site and 3′ primers with HindIII sites and were cloned into pCMV Tag1 (Stratagene) with in-frame NH2 terminus flag tags.

Milton-B (LD33316), -C (LD28289), and -D (AT08952 and AT28797), which differ only in the 5′ untranslated region, were obtained from the Berkeley Drosophila Genome Project (University of California, Berkeley, CA), and their PCR-amplified NH2 termini were substituted for that of milton-A in pCMV Tag1 milton-A. The NH2 termini of these clones were amplified by PCR with 5′ primers containing a BamHI site and a 3′ primer with an XhoI site and cloned into pCMV Tag1 milton-A. pCMV Tag1 milton-C was made similarly, but using NolI at the 5′ instead of BamHI. Human xpress-OIP106 (milton 1) and rat xpress-GRIF-1 (milton 2) were provided by G. Hart (Johns Hopkins University, Baltimore, MD; Iyer et al., 2003). Rat myc-KHC, myc-KHC1-682, myc-KHC1-810, myc-KHC1-891, and HA-KLC constructs were provided by K. Verhey (University of Michigan, Ann Arbor, MI; Verhey et al., 1998). An EST, RE01164, corresponding to D. melanogaster miro (CG5410-PE) was obtained from the Berkeley Drosophila Genome Project and cloned between the BamH1 and NotI sites of pA117 (Shamah et al., 2001), thereby placing a 7′ epitope tag at the NH2 terminus. MiroΔTM was generated by digesting the miro construct with EcoRI and NotI, and then ligating an oligo-encoding a stop codon between these sites.

**Immunostaining**

COS7 and HEK293T cells were cultured in DME supplemented with 10% FCS, l-glutamine, and penicillin/streptomycin. Rat cerebellar neurons were cultured as previously described (Sivasankaran et al., 2004). Cells were transfected with calcium phosphate and immunostained 24–36 h later. In all co-transfection experiments, 1:1 ratios of DNA were used, except for miroΔTM, which was transfected in a 250-fold excess. Immunocytochemistry was performed as previously described (Stowers et al., 2002) and used either anti-milton mAbs 2A108, 4A75, or 5A124, or anti-milton antisera P1–152. Other primary antibodies used in this study include: chick anti-myc (Invitrogen), 9E10 (Santa Cruz Biotechnology, Inc.), chick anti-HA (GTS, Inc.), anti-Xpress (Invitrogen), goat anti-T7 (Bethyl Laboratories, Inc.), anti-cytochrome c oxidase (BD Biosciences), rabbit anti-KLC (a gift from J. Gindhart, University of Richmond, Richmond, VA; Gindhart et al., 1998), mouse anti-HSP60 (Stressgen Bioreagents), and rabbit anti-HA (Novus Biologicals, Inc.). The following fluorescently tagged reagents were used: goat anti-mouse Alexa Fluor 488, goat anti-β-catenin Alexa Fluor 487, donkey anti–mouse Alexa Fluor 487, donkey anti– goat Alexa Fluor 633, and donkey anti–mouse Alexa Fluor 647 (all from Invitrogen), and goat anti–mouse Cy3, horseradish peroxidase Cy5, and donkey anti–rabbit FITC (Jackson Immunoresearch Laboratories).

Cells were imaged at room temperature (25°C) with a 63×, NA 1.4, oil Plan-Apochromat objective lens on a laser-scanning confocal microscope (LSM 510 META/NLO; Carl Zeiss MicroImaging, Inc.) with LSM software 3.2 (Carl Zeiss Micromaging, Inc.). Images were assembled into figures with Photoshop 8.0 (Adobe) using only linear adjustments of contrast and color.

First or third instar larvae were dissected in PBS, fixed in 4% formaldehyde for 20–30 min in PBS, washed three times in PBT (PBS, 0.3% Triton X-100, and 0.5% bovine albumin serum), incubated in PBT (PBT with 5% normal donkey serum) for 30–60 min, and then incubated with 24810 anti-chaoptin (Developmental Studies Hybridoma Bank) at 1:200 overnight in PBT at 4°C. The preparations were washed three times in PBT, then incubated in goat anti–mouse Cy3 (Jackson Immunoresearch Laboratories, Inc.) 1:400 in PBTs, washed three times in PBT and twice in PBS with 0.3% Triton X-100 and mounted in Vectashield (Invitrogen). Confocal images of photoreceptor axons were taken with a 40×, NA 1.3, objective (Carl Zeiss Micromaging, Inc.).

**Materials and methods**

**Visualization of mitochondria**

Mitochondria were visualized in D. melanogaster using transgenic stocks containing mitoGFP (Pilling et al., 2006) and placed under the control of a UAS promoter. Expression in selective tissues was driven by D42-Gal4, which is expressed in a subset of neurons (Fig. 1), or ey-Gal4, which is
precipitating mitohnon with KHC, KLC, or 0.5% Triton X-100 for miro and miiton experiments. Lysates were precluded and antibodies protein A incubated with anti-mitochondion antibodies mAb 9E10 or anti-tubulin, and protein A-Sepharose beads [GE Healthcare] for 2–3 h at 4°C. Immunoprecipitates were separated by SDS-PAGE and transferred to nitrocellulose membranes. For immunodetection, antmitochondion mAb SA124 and anti-mitochondion mAb 2A10 were used at 1:40; rabbit anti-KLC was used at 1:100; mAb 9E10, rabbit anti-HA, and rabbit anti-KHC were used at 1:1,000; and donkey anti-rabbit HRP and goat anti-mouse HRP were used at 1:10,000 (Jackson ImmunoResearch Laboratories, Inc.)

D. melanogaster stocks
The stocks used for these experiments were:


