RESEARCH ARTICLE

A new mode of mitochondrial transport and polarized sorting regulated by Dynein, Milton and Miro

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ABSTRACT

Intrinsic cell microtubule (MT) polarity, together with molecular motors and adaptor proteins, determines mitochondrial polarized targeting and MT-dependent transport. In polarized cells, such as neurons, mitochondrial mobility and transport require the regulation of kinesin and dynein by two adaptor proteins, Milton and Miro. Recently, we found that dynein heavy chain 64C (Dhc64C) is the primary motor protein for both anterograde and retrograde transport of mitochondria in the Drosophila bristle. In this study, we show that a molecular lesion in the Dhc64C allele that reduced bristle mitochondrial velocity generated a variant that acts as a ‘slow’ dynein in an MT-gliding assay, indicating that dynein directly regulates mitochondrial transport. We also showed that in milton-RNAi flies, mitochondrial flux into the bristle shaft, but not velocity, was significantly reduced. Surprisingly, mitochondria retrograde flux, but not net velocity, was significantly decreased in miro-RNAi flies. We thus reveal a new mode of mitochondrial sorting in polarized cell growth, whereby bi-directional mitochondrial transport undertaken exclusively by dynein is regulated by Milton in the anterograde direction and by a Miro-dependent switch to the retrograde direction.

KEY WORDS: Bristle, Drosophila, Dynein, Milton, Miro, Mitochondria

INTRODUCTION

Owing to their roles in ATP production, calcium homeostasis and cell signaling, mitochondria are essential for cellular function and they form a dynamic network within the cell. In highly polarized cells, such as neurons, long-distance transport of mitochondria relies mainly on microtubules (MTs), which ensure the appropriate distribution of the mitochondrial network — a process that is also essential for proper cell function. In neurons of both vertebrates and invertebrates, axonal MTs display a uniform orientation, with their plus-ends facing the axon tip (Baas et al., 1988; Stone et al., 2008). In vertebrate dendrites, MTs present mixed polarity, with equal numbers of plus- and minus-end-out MTs (Baas et al., 1988). However, in invertebrate dendrites, the majority of MTs show uniform polarity, with the minus-ends pointing away from the cell body (Stone et al., 2008). The same is true for Drosophila bristle cells: MT-based mitochondrial transport relies on the plus-end-directed motor protein Kinesin-1, and the minus-end-directed motor cytoplasmic Dynemin-1. In both axons and dendrites, the direction of mitochondrial transport depends on MT polarity (reviewed in Schwarz, 2013).

Motor-based transport of mitochondria is mediated through the actions of several motor-associated adaptor proteins and mitochondrial scaffolding proteins. In Drosophila, the mitochondria adaptor protein Milton is required for kinesin-mediated transport of mitochondria to nerve terminals (Stowers et al., 2002), to photoreceptors (Gorska-Andrzejak et al., 2003) and along the axon (Glater et al., 2006). In mammalian cells, TRAK1 and TRAK2, homologs of the Drosophila Milton protein, are required for the mobility of mitochondria along the axons of hippocampal neurons (Koutsopoulos et al., 2010) and for steering mitochondria to axons and dendrites (van Spronsen et al., 2013). Milton, another mitochondrial motor-adaptor protein, is an atypical mitochondrial GTPase (Fransson et al., 2003), which is required for the transport of mitochondria in Drosophila (Guo et al., 2005) and in mammalian cells (Fransson et al., 2006). It was further demonstrated that Miro forms a complex with Milton and the motor protein Kinesin-1 (Glater et al., 2006) and could directly bind to Kinesin heavy chain (Khc) in a Ca²⁺-dependent manner (Macaskill et al., 2009; Saotome et al., 2008; Wang and Schwarz, 2009).

The highly polarized Drosophila bristle cell represents a unique, dendrite-like cell in which stable MTs are uniformly oriented with their minus-ends pointed toward the bristle tip (Bitan et al., 2012). Thus, in the context of the bristle cell, anterograde transport is defined as MT-minus-end-oriented (i.e. towards the bristle tip) and retrograde transport is defined as MT-plus-end-oriented (i.e. towards the bristle base). We have studied the role of MT motor proteins in mitochondrial long-distance transport in bristle cells and found that in Dynein heavy chain (Dhc64C) mutant bristles, the net velocity of mitochondrial movement in both the anterograde and the retrograde directions was significantly reduced, whereas in Khc mutants, such movement was significantly higher (Melkov et al., 2015). These results suggest that Dhc64C is the primary motor for both anterograde and retrograde fast mitochondria transport, whereas Khc provides a competing force to Dhc64C (Melkov et al., 2015).

To better understand the role of bristle mitochondrial transport, we focused our attention in the present study to the genetic, molecular and biochemical characterization of Dhc64C mutants. We found that mutations in Dhc64C affected bristle morphology and MT and actin organization. We reveal the molecular lesions in three Dhc64C alleles and showed that one of these alleles acts as a ‘slow’ version of dynein in an MT-gliding assay. Surprisingly, examination of the function of two broadly known mitochondrial adaptor proteins, Miro and Milton, showed different roles for each. We found that Miro is preferentially needed for retrograde mitochondrial (i.e. MT plus-end-directed) transport. At the same time, Milton was shown to be responsible for primary polarized mitochondria sorting into the developing bristle cell.

RESULTS

Identification of Dhc64C alleles that affect actin and the MT network during bristle development

The following loss-of-function alleles were used in our study: Dhc64C4-19, Dhc64C602, Dhc64C3-2, Dhc64C6-5, Dhc64C6-12, Dhc64C6-10 and Dhc64C6-1. Whereas Dhc64C4-19 (Gepner et al., 1996) and Dhc64C602 (Li et al., 2008) were reported to be amorphic
alleles, and Dhc64C6-4, Dhc64C6-12 and Dhc64C6-10 (Gepner et al., 1996) were reported to be hypomorphic alleles, the nature of the Dhc64C8-1 and Dhc64C3-2 alleles remained unknown. We therefore first analyzed the lethality of each of the above alleles in combination with a deficiency that removed the 64B13-64C4 region [Df(3L) Exel6102], which included the Dhc64C gene. We found that whereas Dhc64C4-19, Dhc64C902 and Dhc64C3-2 hemizygotes were lethal, Dhc64C6-12, Dhc64C6-1, Dhc64C6-10 and Dhc64C6-6 hemizygotes were viable. However, the affected flies died 4-5 days after eclosion. Based on their viability, we refer to the lethal hemizygous as amorphic alleles and the viable alleles as hypomorphic alleles. Of the hemizygous viable alleles, Dhc64C8-1, Dhc64C8-10 and Dhc64C8-6 but not Dhc64C8-12 presented bristle defects.

Examination of mutant bristles using scanning electron microscopy (SEM) showed these entities to be much shorter than wild-type bristles (Fig. 1). Specifically, measuring the lengths of adult posterior scutellar bristles revealed a mean (±s.d.) length of 213±24 μm (n=6) in Dhc64C mutants, a value that was significantly shorter (F_1,7=353.7, P<0.001) than that in wild-type bristles [339±26 μm (n=15)]. Moreover, closer examination of bristle morphology (Fig. 1B-D) revealed that the upper part (i.e. the first third) of the bristle was very thin and highly twisted (Fig. 1C), and lacked the cuticular structure of ridges and valleys, compared with wild-type bristles (Fig. 1A). The lower part of the bristle closest to the base was wider in the mutant than it was in the wild type and more than 50% of the mutant bristles presented an ectopic extension at the bristle base with a growth direction almost opposite to the main bristle extension (arrows in Fig. 1B,D). Next, rescue experiments were performed to test whether the expression of transgenes encoding HA-Dhc64C (Silvanovich et al., 2003) could rescue the bristle defects found in the Dhc64C hemizygous mutant. We found full rescue of bristle length and no ectopic extension at the bristle base, meaning that the mutation in Dhc64C was responsible for the observed defects. Next, we analyzed the organization of the actin cytoskeleton during pupal development. Phalloidin staining of actin bundles, along with staining of the bristle shaft membrane with antibodies against Dusky-like protein (Dyl) (Fernandes et al., 2010; Nagaraj and Adler, 2012), revealed that in Dhc64C mutant bristles, actin bundles were disorganized in the lower areas of the bristle shaft (Fig. 2D,F,F′). Indeed, digital cross-sections through mutant bristles demonstrated that whereas in wild-type bristles the actin bundles were restricted to the shaft membrane (Fig. 2C′), in Dhc64C mutants, actin bundles were no longer found at the membrane but instead were distributed throughout the bristle cytoplasm (Fig. 2F′). Next, we compared thin transverse sections of bristles from wild type (Fig. 2G,G′; 10 bristles from 3 pupae) and Dhc64C mutants (Fig. 2H,H′; 13 bristles from 3 pupae) 42-48 h after puparium formation (APF), using transmission electron microscopy (TEM). Such analysis revealed that actin bundles could be seen both at the cell membrane and in the bristle shaft in Dhc64C mutant hemizygotes (arrows in Fig. 2H,H′; in all tested bristles), whereas the triangular actin bundles observed in the wild-type bristles were attached to the shaft membrane (red arrows in Fig. 2G,G′). Closer examination of the membrane-bound actin bundles in the mutant (thin black arrows in Fig. 2H,H′) showed them to be much smaller than those in the wild type, with half of the bundles in the mutant being triangular and the other half presenting irregular shapes (red arrows in Fig. 2H,H′). These results corroborate with the actin staining seen in the confocal microscopy study, suggesting that mutations in Dhc64C strongly affect actin organization during bristle development.

Next, we considered the organization of MTs in Dhc64C mutant bristles. To analyze MT organization, developing bristles were stained with antibodies against acetylated tubulin, which stained the stable population of MTs (Fig. 3A-F). In the wild type, acetylated tubulin was abundant along the entire shaft (Fig. 3B-B′). In Dhc64C mutants, MTs were evenly distributed in the lower part of the bristle, (Fig. 3E); however, from the middle of the developing bristle (Fig. 3E′), the MTs were disorganized, with MT-absent areas appearing, as revealed by digital cross-section analysis (Fig. 3E″). The areas lacking MTs expanded gradually towards the bristle tip (Fig. 3E″). Such defects in MT organization were also detected in all three hemizygous alleles studied using confocal microscopy. As described above, whereas the lower part of the Dhc64C mutant bristle contained MTs, a decrease in MT density from the middle part to the tip of the bristle shaft was noted. Closer examination of MT organization by TEM revealed, as described
before (Tilney et al., 2000), that in the WT (10 bristles from 3 pupae were analyzed), the bristle cytoplasm contains a large population of MTs (Fig. 3G and arrows in Fig. 3H). However, in Dhc64C mutants (10 bristles from 3 pupae were analyzed), we found that whereas in five of the analyzed sections MTs were found in the cytoplasm (Fig. 3I, and arrows in Fig. 3J), in the rest of the bristles analyzed, we detected large white areas lacking any organelles (arrowheads in Fig. 3K). Indeed, closer examination of these sections revealed that the entire cytoplasm lacked MTs (Fig. 3L). We believe that sections from Dhc64C mutants bristle, which contain MTs, may represent the lower part of the bristle and the section without MTs may represent the middle to the upper parts of the bristle.

**Dhc64C**<sup>6-1</sup> allele is a ‘slow’ dynein mutant

Of the known EMS-induced Dhc64C alleles, only the lesion in Dhc64C<sup>6-6</sup> has been characterized. Here, a premature stop codon, which results in a truncated product at amino acid 1173, was introduced (Horne-Badovinac and Bilder, 2008). In the present study, we identified the nature of the lesion in Dhc64C<sup>6-1</sup>, Dhc64C<sup>6-6</sup> and Dhc64C<sup>6-10</sup> by sequencing the Dhc64C gene from genomic DNA derived from hemizygous flies. We found that the Dhc64C<sup>6-1</sup> allele contained a specific transversion mutation (A to T), which resulted in an amino acid change, namely D1922V. Closer examination revealed that the aspartic acid at position 1922 that was changed to valine (D1922V) in Dhc64C<sup>6-1</sup> fill the cytoplasm. Scale bars: 2 μm (G,G<sup>*</sup>), 1 μm (H) and 200 nm (H<sup>*</sup>).
significantly slower ($F_{1,132}=711.59$, $P<0.001$). These results suggest that changing the aspartic acid at position 1933 (corresponding to Drosophila D1922), found at the first AAA+ domain, to valine reduced dynein velocity in vitro, probably because of a direct effect on its motor function rather than defects in dynein complex assembly. These results demonstrate that the Dhc64C8-1 allele is a ‘slow’ Dhc mutant.

Regulation of Dhc64C mitochondrial transport is not dependent on the p150Glued dynactin subunit

Having previously shown that dynein is the primary motor protein involved in antero- and retrograde mitochondrial transport (Melkov et al., 2015), we now considered the role of dynein accessory proteins involved in integrated regulation of transport driven by dynein. Dynactin, a dynein activator, is a multi-subunit protein complex that is required for most, if not all, types of cytoplasmic dynein activity in eukaryotes (reviewed in Kardon and Vale, 2009). Dynactin comprises 11 different subunits, including the filament of actin-related protein 1 (Arp1) and the largest subunit, p150 (reviewed in Schroer, 2004). In Drosophila, the Glued (Gl) gene (DCTN1-p150) encodes the homolog of the p150 subunit of the vertebrate dynactin complex (Gill et al., 1991; Holzbaur et al., 1991; Swaroop et al., 1987). To study the function of Glued in bristle development and mitochondrial transport, we specifically reduced Glued levels in the bristle using RNAi or by expressing the dominant-negative form of Glued (Glued-DN), containing only the N-terminal 922 amino acids (Allen et al., 1999; Bielska et al., 2014) using the UAS/Gal4 system. Glued-RNAi (12 flies; 48 bristles were tested) and Glued-DN (3 flies; 12 bristles were tested) flies were viable, yet showed defects in bristle morphology in all tested animals, with the upper part of the bristle being thin and twisted (Fig. 5B-D) compared with wild-type bristles (Fig. 5A). Since both actin and MT network organization was severely affected in Dhc64C mutants bristles (Figs 2,3), we examined the organization of these cytoskeletal components in the bristles of Glued-RNAi and Glued-DN flies. Surprisingly, defects in neither MT nor actin distribution were detected in bristles from Glued-DN flies (Fig. S1D-F).

To test whether Dhc64C mitochondrial transport function was dependent on p150Glued, we quantified mitochondrial transport parameters in the bristles of Glued-DN mutant flies. We found that there were no obvious defects in mitochondrial distribution in bristle cells of Glued-DN flies (Fig. 5F; Movie 4), compared with wild-type cells (Fig. 5E; Movie 3). We also found that there were no defects in the net velocity of anterograde ($2.39\pm2.32\ \mu\text{m/s}$) and retrograde ($2.18\pm1.66\ \mu\text{m/s}$) mitochondrial movement (Table 1). However, a significant shift ($P=0.028$) in the direction of movement was detected in the bristles of Glued-DN mutant flies, with $88.06\pm15.25\%$ of all dynamic mitochondria moving in the anterograde direction and only $11.94\pm15.25\%$ moving in the retrograde direction (Table 1; Fig. 5H). In wild-type bristles, $72\pm19.45\%$ of the measured movement occurred in the anterograde direction and $28\pm19.45\%$ of the measured movement was in the retrograde direction (Melkov et al., 2015; Table 1; Fig. 5G). We also measured the flux of mitochondrial transport [i.e. the number of mitochondrial movements per unit time (100 s) per unit area (100 $\mu\text{m}^2$)] and found that in Glued-DN flies, there was a significant reduction ($P<0.001$) in the retrograde flux to $0.24\pm0.29$, but not anterograde flux ($2.74\pm
1.84), compared with wild type, where retrograde mitochondria flux was 0.92±0.68 and anterograde flux was 2.39±1.45 (Table 1). Since it was shown that motor-based transport of mitochondria is mediated through the action of several adaptor proteins, we analyzed the functions of two well-known mitochondrial adaptor proteins, Milton and Miro. To study the function of Milton in mitochondrial transport, we specifically reduced Milton levels in the bristle by RNAi, using the UAS/Gal4 system. We found that Milton-RNAi flies were viable, with no obvious defects in bristle morphology in all tested animals (Fig. 6A; 4 flies, 16 bristles were tested). However, upon following mitochondrial mobility in the mutant flies, we noted defects in polarized sorting of mitochondria into bristle cells. We found that early in bristle cell differentiation, Milton is required for polarized sorting of mitochondria into bristle cells.

**Fig. 4. Identification of the nature of the genetic lesion in Dhc64C8-1, Dhc64C6-6 and Dhc64C6-10 alleles.** (A) Schematic showing the overall structure of the dynein heavy chain protein. The protein is divided into four domains, namely the tail, which is the cargo-binding domain, the head (motor), which is the site of ATP hydrolysis (containing six AAA+ domains), the linker, which is the mechanical amplifier, and the stalk, which is the microtubule-binding domain (MTBD). In the Dhc64C8-1 allele, a D1922V change was noted, with this residue being found in the first AAA+ domain. This Asp is conserved from yeast to Drosophila (box 1). In the Dhc64C6-6 and Dhc64C6-10 alleles, a W1527R change was detected, with this residue being found in the linker domain. This Trp is also conserved from yeast to humans (box 2). Another mutation was found in the Dhc64C6-6 allele, leading to a G1147C change, with this residue being found in the tail domain and conserved from yeast to humans (box 3). (B) Size-exclusion chromatography traces for the wild-type recombinant dynein complex (red) and mutant recombinant dynein complex (DYNC1H1D/V) (black). Both complexes eluted at the same volume. (C) Coomassie Blue-stained SDS-PAGE of the purified recombinant dynein complex. The inset highlights the 10-15 kDa range from a gel with better low molecular mass separation in which bands corresponding to the different light chains can be discriminated. All dynein complex subunits are present in the wild-type (lane A) and DYNC1H1D/V complexes (lane B). (D-G) Stills from an MT-gliding assay with immobilized recombinant human dynein. D,E, wild-type recombinant dynein complex; F,G, DYNC1H1D/V recombinant human dynein. In D,F, t1=0; in E,G, t2=40 s. Red arrowheads mark the ends of the MTs. The gliding distance was much smaller with DYNC1H1D/V than with the wild type. (H,I) Representative kymograph of wild-type DYNC1H1 complex (H) and mutant DYNC1H1D/V complex (I). Time is on the y-axis, with the vertical bar indicating 100 s. The calculated gliding MT velocities are shown in each kymograph.
development in the wild type (i.e. 32 h APF), mitochondria were evenly distributed within the bristle shaft (Fig. 6B). In contrast, mitochondria were completely absent from the bristle shaft in milton-RNAi flies (Fig. 6B′). Later in development (i.e. 36-39 h APF), a small amount of unequally distributed mitochondria could be detected within the bristle shafts of the mutant flies (Fig. 6C′,D′). Since it was shown that Milton may interact with Dynein in sorting mitochondria into dendrites (van Spronsen et al., 2013), we tested...
whether mitochondrial distribution was likewise altered during bristle development in *Dhc64C* flies. We found no defects in mitochondrial distribution during development in *Dhc64C* flies (Fig. 6B, C, D). Next, we quantified mitochondrial transport parameters in *milton-RNAi* mutant fly bristles (Movie 5) and found no significant difference in mitochondrial net velocity (Table 1; anterograde velocity, 2.45±1.66 and retrograde, 2.21±1.46 μm/s). Unexpectedly, we found no significant defects in the proportion of directional movement (anterograde: 72.34±18.92%; retrograde: 27.66±18.92; Fig. 5I), compared with the wild type (Movie 3). However, we did detect a significant reduction in both anterograde (1.00±0.85; \( P = 0.009 \)) and retrograde (0.38±0.34; \( P = 0.0063 \)) mitochondrial flux, compared with that in wild-type flies (Table 1). Thus, both the temporal delays in mitochondrial entry into the developing cell and the overall flux decreases observed suggest that Milton is involved in the early initiation of mitochondrial transport or is responsible for the polarized sorting of mitochondria.

**Miro is required for retrograde motility of mitochondria during bristle development**

It is broadly accepted that the protein Milton functions together with a second protein, Miro, to regulate mitochondrial transport (reviewed in Schwarz, 2013). As we found that Milton is required for sorting mitochondria into the bristle shaft, we now tested whether this function also required Miro. Accordingly, we first tested whether mutations in *miro* affected bristle development. Examination of bristle morphology from *dMiroB682/dMiroSd32* flies shows no detectable morphological defects. For comparison with the same region in wild-type flies, see Fig. 5A. (B) Confocal projection from a developing bristle expressing UAS-Mito-GFP from wild-type flies, ∼32 h APF. The young bristle cell at the beginning of the elongation process already fulfilled with mitochondria is seen. (B′) An elongating bristle from *neur-Gal4-UAS-Milton-RNAi44477* flies at ∼32 h APF shows a lack of mitochondria inside the developing young bristle. The yellow dashed line here and in the following images marks the border of the cell. (B″) Confocal projection from the bristle from *Dhc64C8-1/Dhc64C4-19* flies ∼32 h APF shows no obvious defects in mitochondrial distribution. (C) Confocal projection from a wild-type bristle at ∼36 h APF shows a dense mitochondria network filling the cell. (C′) Confocal projection from an elongating bristle from a *neur-Gal4-UAS-Milton-RNAi44477* at ∼36 h APF shows decreased mitochondria density. (C″) Confocal projection from the elongating bristle from a *Dhc64C8-1/Dhc64C4-19* by ∼36 h APF shows a pattern similar to wild-type mitochondrial distribution. (D) Confocal projection from a wild-type bristle at ∼39 h APF, with the cell at an advanced elongation stage still showing mitochondria evenly distributed from the base to the tip, in contrast to what was seen in *neur-Gal4-UAS-Milton-RNAi44477* flies at the same developmental stage (D′), demonstrating defective mitochondrial distribution and delayed mitochondria entry into the elongating bristle. (D″) Confocal projection from a *Dhc64C8-1/Dhc64C4-19* bristle at ∼39 h APF shows no detectable defects in the mitochondrial localization pattern. Scale bars: 10 μm.

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**Table 1. Mitochondrial transport parameters in *Drosophila* bristles**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. of moving mitochondria</th>
<th>Directionality proportion (%)</th>
<th>Velocity (μm/s)</th>
<th>Flux</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anterograde</td>
<td>Retrograde</td>
<td>Anterograde</td>
<td>Retrograde</td>
</tr>
<tr>
<td>Wild type (N=7; n=7)</td>
<td>163</td>
<td>57</td>
<td>238</td>
<td>26</td>
</tr>
<tr>
<td>Glued-DN (N=5; n=10)</td>
<td>74±5</td>
<td>26±2</td>
<td>88.06±15.25*</td>
<td>11.94±15.25*</td>
</tr>
<tr>
<td><em>milton-RNAi</em> (N=6; n=12)</td>
<td>2.04±1.06</td>
<td>2.08±0.19</td>
<td>2.39±2.32</td>
<td>2.18±1.66</td>
</tr>
<tr>
<td><em>miro-RNAi</em> (N=5; n=11)</td>
<td>2.39±1.45</td>
<td>0.92±0.68</td>
<td>2.74±1.84</td>
<td>0.24±0.29*</td>
</tr>
</tbody>
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The leading moving edge of each mitochondrion was marked in every frame of each movie taken from animals of each genotype. Directionality proportions, velocity and flux values reflect mean±s.d. Mutant parameters found to be significantly different from those of the wild type by nested ANOVA followed by a post hoc Tukey test (\( P < 0.001 \)) are marked with an asterisk.

N, number of pupae; n, number of bristles.
transheterozygotes, which died at late pupal stage before eclosion, revealed no significant phenotypic defects, with overall bristle length being normal (Fig. 7B,C). Indeed, only mild structural cuticular morphological aberrations were found in all tested bristles, with the characteristic parallel ridge surface morphology being affected in the middle part of the bristle (Fig. 7C; 16 bristles from 4 adult flies). We also downregulated miro levels in the bristles using Miro-RNAi106683 and found, as in the mutants, no defects in bristle lengths (Fig. 7D), although a slightly disrupted cuticular surface in the upper bristle regions was noted in all tested bristles (Fig. 7E; 16 bristles from 4 adult flies). Since there was a difference between the miro transheterozygote and Miro-RNAi106683 knockdown adult bristle phenotypes, we used a second RNAi line (UAS-Miro-RNAi106683; Iijima-Ando et al., 2012) to confirm that the cuticular phenotype in Miro-RNAi106683 was indeed due to a loss of Miro and not the result of off-target effects. We found that downregulation of miro levels in the bristle using UAS-Miro-RNAi106683 (15 bristles from 6 adult flies; Fig. 7F,G) led to the identical cuticular phenotype as seen in the Miro-RNAi106683 flies (Fig. 7G). Next, we examined mitochondrial distribution in Miro-RNAi106683 flies and found that in contrast to the Milton-dependent mitochondrial phenotype, Miro-RNAi106683 flies presented no defects in mitochondrial distribution (Fig. 7H, Movie 6). Moreover, no obvious defects in MTs or actin organization were seen in the bristles of Miro-RNAi106683 flies (Fig. S1G-I). Next, we measured mitochondrial movement in Miro-RNAi106683 flies (Table 1 and Movie 6) and found no significant differences in either the anterograde (−2.09±1.36 μm/s) or retrograde (−1.45±0.54 μm/s) direction compared with the wild type. However, we detected a significant reduction (P=0.001) in retrograde (−0.19±0.16) but not anterograde mitochondrial flux (−2.68±1.19). Finally, a significant reduction (P=0.032) in the proportion of the retrograde-moving mitochondria was found in Miro-RNAi106683 (−7.72±6.81%) (Table 1 and Fig. 5J). Similar defects in mitochondria directionality and flux measurement were also detected using UAS-Miro-RNAi106683 (Table S1).

**DISCUSSION**

**Dynein is the primary motor in both anterograde and retrograde bristle mitochondrial transport**

Long-distance mitochondrial transport occurs mainly on MTs and is promoted by motor proteins. It was shown that kinesin and dynein motors drive mitochondrial transport in a manner reflecting microtubule polarity (reviewed in Schwarz, 2013). Accordingly, we found that whereas mutation in Dhc64C significantly reduced mitochondrial net velocity in both the antero- and the retrograde directions, mutations in Ahe enhanced such velocities. These results further suggested that dynein is the primary motor for anterograde and retrograde bristle mitochondrial transport. In the current study, we provided additional evidence for the direct role of dynein in bristle mitochondrial transport by biochemically characterizing the effects of a mutation found in Dhc64Cα-1 allele that was used in our previous study. We first identified the molecular lesion in the Dhc64Cα-1 allele and localized it to the first AAA+ domain. Among the six AAA+ domains in the protein, hydrolysis in the first such domain mainly provided the energy needed for dynein motility. The affected residue corresponds to D1933 in human DYNC1H1, and it is found in the loop between β-strand S2 and helix H2 in the luminal-facing portion of the AAA+ domain. In an MT-gliding assay using fully recombinant wild-type human dynein complex and mutant dynein complex (DYNC1H1ΔV), we demonstrated that this...
mutation significantly reduced MT-gliding activity, similar to the reduction in bristle mitochondrial net velocity found in our previous study (Melkov et al., 2015). Thus, our genetic and biochemical characterization revealed that the protein encoded by Dhc64C\(^{8-1}\) is a ‘slow’ dynein mutant. The fact that this variant is a ‘slow’ dynein mutant, along with our finding that the net velocity of mitochondrial transport in both anterograde and retrograde directions is highly reduced in the presence of this allele (Melkov et al., 2015), provides further support for the notion that Dhc64C directly regulates anterograde and retrograde mitochondrial transport along the bristle shaft.

**Dhc64C bristle phenotypes are probably not due to defects in mitochondrial transport**

Previously, it was shown that mutations connected to mitochondrial function affect bristle morphology, resulting in the appearance of short and thin bristles (Mourikis et al., 2006). Our analysis of the defects in mitochondrial transport in Dhc64C bristle cells led us to further investigate the nature of the defects in the bristles of such mutants. We demonstrated that mutations in Dhc64C resulted in the production of shorter and thinner bristles (at least in the upper part of the shaft). Moreover, we found that both actin bundles and MT networks were dramatically disorganized in flies containing the mutant alleles. We found that more than 50% of the bristles presented defects in their growth direction. We also found that bristle morphology was strongly affected in Glued-RNAi and Glued-DN flies but surprisingly, no internal MT or actin organization defects were detected. However, examination of mitochondrial transport in Glued mutant bristles revealed that, in contrast to Dhc64C, this gene is not involved in the control of the mitochondrial velocity but rather serves a role in mitochondrial retrograde sorting. Examination of miro mutant bristles, the product of which is also involved in mitochondrial retrograde sorting, showed only mild bristle morphology defects. Unexpectedly, we found that in miltion-RNAi mutant flies, where mitochondrial distribution in the shaft is severely affected as a result of defects in polarized sorting into the bristle shaft, wild type-like bristles were observed. It was also shown that although mitochondria were absent in axons and synapses in miltion mutants, these structures presented normal general architecture, possessing microtubules, synaptic vesicles and active zone specializations (Gorska-Andrzejak et al., 2003; Stowers et al., 2002). Since affecting the mitochondrial sorting machinery has little or no effect on bristle morphology, it would thus appear that the observed defects in Dhc64C bristle mutants are probably not due to defects in mitochondrial transport. Proper mitochondrial function, rather than mitochondrial transport or sorting, is crucial for the normal cell elongation process.

**milton and miro play different roles in mitochondrial sorting**

Transport of mitochondria in neurons, and possibly in most animal cells, requires a motor/adaptor complex (Brickley et al., 2005; Brickley and Stephenson, 2011; Fransson et al., 2003; Glater et al., 2006; Guo et al., 2005; Stowers et al., 2002). The core of this complex consists of three proteins, namely Khc, Miro and Milton. Recently, it was shown that dynein also interacts with Milton and Miro (van Spronsen et al., 2013). Milton was first identified as a protein that is required for Drosophila axonal transport of mitochondria to synapses (Stowers et al., 2002). Miro, an atypical mitochondrial GTPase (Fransson et al., 2003), was later identified as being encoded by a distinct gene in Drosophila associated with mitochondrial transport in axons (Guo et al., 2005). Later, it was shown that Milton and Miro form a protein complex that directly binds Khc to mitochondria (Glater et al., 2006). The mechanism by which Milton-Miro regulates mitochondrial mobility is by the binding of Khc to Miro via Milton to activate this complex in a Ca\(^{2+}\)-dependent manner, although the binding of Miro directly to Khc inactivates the motor by preventing its binding to MTs (Wang and Schwarz, 2009). Recently, it was shown the mammalian Milton homologs TRAK1 and TRAK2 steer mitochondrial transport into axons and dendrites by regulating both Khc and dynein motor proteins (van Spronsen et al., 2013). In the present study, we have shown for the first time that Milton and Miro assume different roles in regulating mitochondrial bristle transport. We found that mutations in these genes have different effects on mitochondrial transport. Whereas in miltion-RNAi, mitochondria failed to enter the bristle shaft and presented defects in their anterograde mobility, upon introduction of miro RNAi, retrograde mitochondrial transport was severely affected. Based on our results, we suggest that dynein serves as the primary motor for mitochondrial bristle transport, and that miltion and miro are not required for mitochondrial transport per se but rather regulate mitochondrial polarized sorting, with miltion being required for anterograde transport and miro being needed for retrograde mobility.

**MATERIALS AND METHODS**

**Drosophila stocks**

Oregon-R was used as a wild-type control. The following mutant and transgenic flies were used: Dhc64C\(^{19-10}\)/TM6B, Dhc64C\(^{8-1}/TM6B, Dhc64C^{8-2}/ TM6B, Dhc64C^{6-4}/TM6B, Dhc64C^{10-10}/TM6B (Gepner et al., 1996), FRT79D Dhc64C\(^{10-10}/TM6B. Li et al., 2008), UAS-mito-HA-GFP (Pilling et al., 2006), Df-64B13-64C4/TM6B (Bloomington, #7581), UAS-Milton-RNAi (Bloomington, #44477), UAS-p150-delta96B (Bloomington, #51645), UAS-p150-RNAi (Bloomington, #24760), UAS-Miro-RNAi\(^{9665}\) (Vienna Drosophila RNAi Center, #100683), UAS-Miro-RNAi\(^{9682}\) (ijijima-Ando et al., 2012), dMiro\(^{9682}\) and dMiro\(^{9665}\) (Guo et al., 2005). Bristle expression was induced under the control of the near-Gal4 or sca-Gal4 driver.

**Dissection and preparation of pupae for live imaging**

After removing the pupal case, the pupae were dissected as previously described (Tilney et al., 1998; Melkov et al., 2015).

**Bristle Phalloidin and antibody staining**

The procedures used for fixation and staining were described previously (Guild et al., 2003; Melkov et al., 2015). Confocal images were taken using an Olympus FV1000 laser scanning confocal microscope and are shown here as 2-projections in a few optical frames that together covered the bristle cell. Primary antibodies used were anti-acetylated tubulin mouse monoclonal antibodies (1:250; Sigma, T7451) and anti-Dyl (Dusky-like) rabbit polyclonal antibodies (1:250) (Fernandes et al., 2010). Cy3-conjugated goat anti-mouse (1:100; Jackson ImmunoResearch) and Alexa Fluor 488-conjugated goat anti-rabbit (1:100; Molecular Probes) secondary antibodies were used. For actin staining, we used Oregon Green 488- or Alexa Fluor 568-conjugated Phalloidin (1:250; Molecular Probes).

**Transmission and scanning electron microscopy sample preparation**

Pupae were dissected and prepared for transmission electron microscopy, as previously described (Melkov et al., 2015). The slices were stained with uranyl acetate and lead citrate and visualized with a JEM 1230 (JEOL) transmission electron microscope at 120 kV. For scanning electron microscopy, samples were fixed in 4% paraformaldehyde, 2% glutaraldehyde in 0.1M phosphate buffer, pH 7.4, for 1 h at room temperature and post-fixed for 1 h in 1% OsO\(_4\) in the same buffer. After washing with PBS, the material was dehydrated through an ethanol series and critical point dried. The samples were then mounted on adhesive stubs and sputter coated with gold palladium. Images were obtained with a JEOL Sigma 800 electron microscope using a 1 kV accelerating voltage.
This mutation corresponds to the product of the expression and purification of wild-type and mutant dynein were conducted. Recombinant proteins was conducted as described (Schlager et al., 2014). Expression and purification of wild-type and mutant dynein complex in Melkov et al. (2015). Velocity was log transformed and an angular square-root transformed to correct for the deviation from normality.

Supplementary information

A.M. designed the study, performed the experiments, analyzed the data and wrote the paper. R.B. performed the experiments, Y.A. analyzed the data. U.A. conceived and supervised the study, performed the experiments, analyzed the data and wrote the paper.

References


Supplemental Figure 1. *Glued* and *Miro* mutants show no defects in actin and stable microtubule organization. Confocal projections of bristles from wild type, scale bar 20 μm (A-C), *neur-Gal4-UAS-p150-delta96B*\textsuperscript{51645}, scale bar 20 μm (D-F) and *neur-Gal4-UAS-Miro-RNAi*\textsuperscript{106683}, scale bar 10 μm (G-I) pupae stained with Oregon green-phalloidin (green) and with anti-acetylated-tubulin antibodies (red). No obvious defects in stable microtubule localization pattern were detected in both mutants.
Supplemental Movie 1 - Microtubule gliding assay with wild type (DYNC1H1) immobilised recombinant human dynein.

Representative time-lapse movie of gliding of 543-labelled microtubules by surface-immobilised GFP-dynein with GFP antibodies (GFP channel not shown).

This assay was performed in 30 mM HEPES, 5 mM MgSO4, 1 mM DTT, 1 mM EGTA, 40 μM taxol, 1 mg/ml α-casein, 2.5mM ATP, pH 7.0).
Supplemental Movie 2 - Microtubule gliding assay with mutant (DYNC1H1<sup>D/V</sup>) immobilised recombinant human dynein.

Representative time-lapse movie of gliding of 543-labelled microtubules by surface-immobilised GFP-dynein with GFP antibodies (GFP channel not shown).

This assay was performed in 30 mM HEPES, 5 mM MgSO4, 1 mM DTT, 1 mM EGTA, 40 μM taxol, 1 mg/ml α-casein, 2.5 mM ATP, pH 7.0).
Supplemental Movie 3. Mitochondria tracking in a developing wild type bristle cell.

Tracking of the mitochondrial reporter *Neur-Gal4-Mito-GFP* in the middle part of a developing wild type bristle cell, using time-lapse confocal microscopy.

Tracking of the mitochondrial reporter Neur-Gal4-Mito-GFP in the middle part of a developing neur-Gal4-UAS-Miro-RNAi106683 bristle cell, using time-lapse confocal microscopy.

Tracking of the mitochondrial reporter *Neur-Gal4-Mito-GFP* in the middle part of a developing *neur-Gal4-UAS-Milton-RNAi* bristle cell, using time-lapse confocal microscopy. An overall decrease in mitochondrial density was seen, although no significant change in net mitochondrial velocity was noted.

Tracking of the mitochondrial reporter Neur-Gal4-Mito-GFP in the middle part of a developing neur-Gal4-UAS-p150-delta96B51645 bristle cell, using time-lapse confocal microscopy.
Supplemental table 1. Mitochondrial movement parameters in *Miro-RNAi*mut. Mutant parameters found to be significantly different from those of the wild type are marked with an asterisk.

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<tr>
<th>Table 1. Mitochondrial transport parameters in <em>Drosophila</em> bristles</th>
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<tr>
<td><strong>Movement directionality</strong></td>
</tr>
<tr>
<td>No. of pupae</td>
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<tr>
<td>No. of bristles</td>
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<tr>
<td>No. of moving mitochondria</td>
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<tr>
<td>Directionality proportion (%)</td>
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<td>Velocity (μm/sec)</td>
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