Microtubule binding by dynactin is required for microtubule organization but not cargo transport

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Dynactin links cytoplasmic dynein and other motors to cargo and is involved in organizing radial microtubule arrays. The largest subunit of dynactin, p150^{glued}, binds the dynein intermediate chain and has an N-terminal microtubule-binding domain. To examine the role of microtubule binding by p150^{glued}, we replaced the wild-type p150^{glued} in *Drosophila melanogaster* S2 cells with mutant ΔN-p150 lacking residues 1–200, which is unable to bind microtubules. Cells treated with cytochalasin D were used for analysis of cargo movement along microtubules. Strikingly, although the movement of both membranous organelles and messenger ribonucleoprotein complexes by dynein and kinesin-1 requires dynactin, the substitution of full-length p150^{glued} with ΔN-p150^{glued} has no effect on the rate, processivity, or step size of transport. However, truncation of the microtubule-binding domain of p150^{glued} has a dramatic effect on cell division, resulting in the generation of multipolar spindles and free microtubule-organizing centers. Thus, dynactin binding to microtubules is required for organizing spindle microtubule arrays but not cargo motility in vivo.

Introduction

All cells maintain nonrandom distributions of cytoplasmic components, including membranous organelles and macromolecular RNA or protein complexes. This cytoplasmic organization is accomplished by a concerted effort of molecular motors, which are proteins that transport cargo along microtubules or actin filaments using the energy of ATP hydrolysis (Schliwa and Woehlke, 2003; Vale, 2003; Mallik and Gross, 2004). The docking of motors to their specific cargoes is essential for proper cargo distribution. Several docking proteins that are essential for binding cytoplasmic dynein, kinesin, or myosin motors to cargo have been described recently (Fukuda et al., 2002; Kamal and Goldstein, 2002; Karcher et al., 2002; Wu et al., 2002). The most versatile and ubiquitous adaptor is the dynactin complex (Gill et al., 1991; Holleran et al., 1998; Karki and Holzbaur, 1999; Schroer, 2004). The main function of dynactin is to facilitate the attachment of cytoplasmic dynein to its cargo (Karki and Holzbaur, 1995; Vaughan and Vallee, 1995). In addition, dynactin can function as an adaptor for at least two motors of the kinesin superfamily, heterotrimeric kinesin-2 (Deacon et al., 2003) and mitotic kinesin Eg-5 (Blangy et al., 1997). Dynactin can also act independently of cytoplasmic dynein to anchor microtubules at the centrosome (Quintyne et al., 1999; Quintyne and Schroer, 2002) and organize radial microtubule arrays (Askham et al., 2002).

The dynactin complex consists of two morphologically distinct structural domains: a rod-shaped domain that binds to the cargo and an extended projection that mediates an interaction with cytoplasmic dynein and microtubules. The rod-shaped part consists of an Arp-1 filament and actin-capping proteins, whereas the projection is formed by a homodimer of a p150^{glued} protein subunit. These two parts of the dynactin complex are bridged by the p50 subunit dynamitin. p150^{glued} interacts with other subunits of the dynactin complex through its C terminus and with cytoplasmic dynein and other motors through its coiled-coil domains (Schroer, 2004).

Remarkably, in addition to providing a platform for motor binding, p150^{glued} has the ability to interact with microtubules independently of cytoplasmic dynein. The microtubule-binding region of p150^{glued} is localized at the extreme N terminus and consists of a CAP-Gly (cytoskeleton-associated protein glycine rich) domain and a basic region, both of which are positioned within the first 200 amino acid residues (Waterman-Storer et al., 1995; Vaughan et al., 2002; Culver-Hanlon et al., 2006).

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Abbreviations used in this paper: dFMRP, *Drosophila* homologue of the fragile X mental retardation protein; DHC, dynein heavy chain; KHC, kinesin heavy chain; mRFP, monomeric red fluorescent protein; mRNP, messenger RNP; MTOC, microtubule-organizing center; UTR, untranslated region.

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Analysis of p150^{glued} isoforms in the mammalian brain showed that in addition to the full-length p150^{glued}, neurons express an alternatively spliced 135-kD isoform lacking the microtubule-binding domain (Tokito et al., 1996). This suggests that this domain could be dispensable for at least some of the dynactin functions in nondividing cells.

In vitro motility analysis using beads coated with a mixture of cytoplasmic dynein and dynactin demonstrated that dynactin could function as a processivity factor for dynein (King and Schroer, 2000), presumably by providing an extra site for microtubule binding and, thus, preventing cargo dissociation from microtubules (Waterman-Storer et al., 1995; Culver-Hanlon et al., 2006; Kobayashi et al., 2006). Another function of the microtubule-binding domain of p150^{glued} is to localize the dynactin complex to the plus ends of growing microtubules (Vaughan et al., 2002). p150^{glued} is a member of a family of microtubule plus end–binding proteins (Akhmanova and Hoogenraad, 2005) and colocalizes with other proteins of this class such as CLIP-170 and EB1 to the plus ends of growing microtubules (Vaughan et al., 1999; Ligon et al., 2003; Lansbergen et al., 2004). Its binding affinity to microtubules is regulated by phosphorylation (Vaughan et al., 2002). It has been postulated that the accumulation of p150^{glued} at the plus ends of microtubules facilitates the loading of retrograde cargo on microtubules (Vaughan, 2005b) and linking microtubule plus ends to specific sites, such as mitotic kinetochores and the cell cortex (Mimori-Kiyosue and Tsukita, 2003).

Both the tip binding and enhancement of motor processivity by dynactin require the N terminus of p150^{glued}. However, the existence of the shorter p135 isoform of p150^{glued}, which lacks the microtubule-binding motif, suggests that the dynactin complex can perform at least some of its functions even without this microtubule-binding activity. In this study, we examine the role of the microtubule-binding domain of p150^{glued} in the cargo transport and organization of microtubules. In cultured *Drosophila melanogaster* S2 cells, we replaced the full-length p150^{glued} protein with a truncated form lacking the microtubule-binding domain. We then examined effects of the deletion of the microtubule-binding domain on cargo transport (membranous organelles and mRNA–protein complexes) and the organization of microtubules. To eliminate the effect of the actin-based component on transport, we treated cells with cytochalasin D. Our results demonstrated that truncation of the first 200 amino acid residues from p150^{glued} eliminated its binding to microtubules but had no effect on the rate, processivity, or step size of cargo transport by either kinesin-1 or cytoplasmic dynein. However, truncation of the microtubule-binding domain resulted in defects in organization of the mitotic spindles, including the formation of multipolar spindles and free microtubule-organizing centers (MTOCs). Thus, we conclude that the microtubule-binding domain of p150^{glued} is not required for microtubule-dependent transport but is essential for the proper organization of radial microtubule arrays.

Results

Generation of cell lines and RNAi procedure

To replace the wild-type p150^{glued} with a truncated form, we generated S2 cell lines expressing a fusion protein of monomeric red fluorescent protein (mRFP; Campbell et al., 2002) or EGFP with the N terminus of either full-length p150^{glued} or p150^{glued} with a deletion of residues 1–200 (ΔN-p150^{glued}). Stable cell lines were selected by hygromycin. We then treated cells with double-stranded RNA corresponding to the 3′ untranslated region (UTR) of p150^{glued} mRNA to deplete the endogenous protein.

Western blotting with an antibody that recognizes the C-terminal fragment of p150^{glued} showed that in addition to the endogenous protein, stable cell lines expressed new proteins with the molecular weights expected for fusions of either full-length p150^{glued} or ΔN-p150^{glued} tagged with mRFP (Fig. 1, lanes 1 and 3) or EGFP (not depicted). The antibody generated against residues 1–200 of p150^{glued} does not recognize ΔN-p150^{glued} (Fig. 1, lane 7), demonstrating that our construct is indeed lacking its N terminus.

As mentioned above, to deplete endogenous p150^{glued}, we treated cells with a double-stranded RNA corresponding to the 3′ UTR of p150^{glued} mRNA. As shown in Fig. 1 (lanes 2, 4, 6, and 8), such treatment dramatically reduced the level of endogenous p150^{glued} but did not affect the expression of mRFP-tagged p150 fusion proteins, as mRNAs encoding these proteins do not have the 3′ UTR. Serial dilutions of samples demonstrated that the level of endogenous p150^{glued} in RNAi-treated cells dropped below 10% of the control untreated cells (unpublished data). Thus, by using a combination of the stable expression of tagged p150^{glued} constructs and RNAi-mediated knockdown of the endogenous p150^{glued}, we can replace the endogenous protein with mRFP or with EGFP-tagged p150^{glued} or ΔN-p150^{glued}.

ΔN-p150^{glued} forms the dynein–dynactin complex

To examine whether truncation of the microtubule-binding domain affected the ability of p150^{glued} to form a dynactin complex and interact with cytoplasmic dynein, we analyzed the sedimentation behavior of the dynein–dynactin complex in sucrose density gradients (Schroer and Sheetz, 1991). We performed these

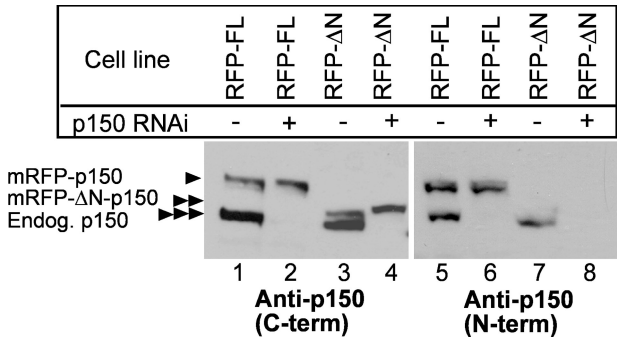

Figure 1. **Western blots of extracts of S2 cells expressing mRFP-p150^{glued} or mRFP–ΔN-p150^{glued}.** mRFP-p150^{glued}, lanes 1, 2, 5, and 6; mRFP–ΔN-p150^{glued}, lanes 3, 4, 7, and 8. Endogenous p150^{glued} was depleted by using RNAi against the 3′ UTR of p150^{glued} mRNA (lanes 2, 4, 6, and 8). Lanes 1–4 were probed with an antibody against the C-terminal fragment of p150^{glued}; lanes 5–8 were probed with an antibody against the N-terminal fragment of p150^{glued}. Positions of mRFP-p150^{glued}, mRFP–ΔN-p150^{glued}, and endogenous p150^{glued} bands are marked by single, double, and triple arrowheads, respectively.

experiments using wild-type S2 cells and cells expressing EGFP-tagged p150^{glued} or ΔN-p150^{glued}. Endogenous p150^{glued} was depleted from cells expressing EGFP-tagged proteins by RNAi. To monitor the sedimentation behavior of p150^{glued}, we used either an N-terminal p150^{glued} antibody (for untransfected cells) or an antibody against EGFP (for transfected cells). The distribution of cytoplasmic dynein in gradient fractions was probed using an anti–dynein heavy chain (DHC) antibody. Western blot analysis of the fractions demonstrated that endogenous p150^{glued}, EGFP- p150^{glued}, and EGFP–ΔN-p150^{glued} have identical sedimentation profiles in sucrose gradients, with a peak in fractions 18–20 that coincides with the peak of cytoplasmic dynein (Fig. 2 A). Kinesin heavy chain (KHC) was probed as a control protein that does not sediment with DHC. These results indicate that neither truncation of the microtubule-binding domain nor fusion with EGFP affects the ability of p150^{glued} to incorporate into the dynactin complex.

To further confirm formation of the dynactin complex by truncated p150^{glued}, we performed an immunoprecipitation assay using anti-EGFP antibody to pull down EGFP-p150^{glued} or EGFP–ΔN-p150^{glued} and probed the precipitates for other dynactin subunits. Fig. 2 B shows that both p50-dynamitin and Arp1 were detected in the precipitates from cells expressing EGFP-p150^{glued} or EGFP–ΔN-p150^{glued}, but not from untransfected S2 cells.

Analysis of these precipitates with the DHC antibody demonstrates the presence of cytoplasmic dynein in the samples precipitated with EGFP antibody from cells expressing EGFP-p150^{glued} or EGFP–ΔN-p150^{glued}. DHC was not detected in the anti-EGFP immunoprecipitates from untransfected S2 cells or in precipitates with a preimmune serum. We conclude that ΔN-p150^{glued} incorporates into the dynactin complex and that this complex interacts with cytoplasmic dynein.

Microtubule binding depends on the N-terminal domain of p150^{glued}

To examine microtubule binding by EGFP-p150^{glued} or EGFP–ΔN-p150^{glued}, we performed a pelleting assay with microtubules in vitro and colocalization studies with microtubules in S2 cells. For microtubule pelleting assays, we expressed recombinant proteins that contain either amino acid residues 1–600 or 200–600 of p150^{glued} fused to EGFP and His_6 tags. Both proteins were purified by using a Talon affinity column, incubated with taxol-stabilized microtubules, and pelleted by centrifugation through a glycerol cushion. As shown in Fig. 3 A, p150^{glued} (amino acids 1–600) bound to microtubules, whereas the truncation of 200 residues from the N terminus abolished microtubule binding. These results confirm previous studies demonstrating that the microtubule-binding domains of p150^{glued} are localized within residues 1–160 of the protein (Waterman-Storer et al., 1995; Culver-Hanlon et al., 2006).

For in vivo analysis of microtubule binding, we depleted endogenous p150^{glued} by RNAi and transiently transfected cells with either EGFP-p150^{glued} or EGFP–ΔN-p150^{glued} constructs. Cells were extracted with Triton X-100 to remove soluble proteins, fixed in methanol, and double stained for microtubules and EGFP. As shown in Fig. 3 B, EGFP-p150^{glued} decorated cytoplasmic microtubules. On the other hand, in agreement with in vitro results, EGFP–ΔN-p150^{glued} did not show any microtubule binding. In addition, both EGFP-p150^{glued} and EGFP–ΔN-p150^{glued} were found in small clusters in the cytoplasm. We do not know the nature of these clusters, but they are probably identical to the cortical p150^{glued}/APC clusters previously observed by Reilein and Nelson (2005) in association with microtubules in MDCK cells.

The interaction of p150^{glued} and ΔN-p150^{glued} with microtubules was also examined in stable cell lines using a spinning disc confocal microscope. Similar to fixed cells, EGFP- p150^{glued} in live S2 cells was localized along microtubules and formed clusters that move bidirectionally along microtubules (Video 1, available

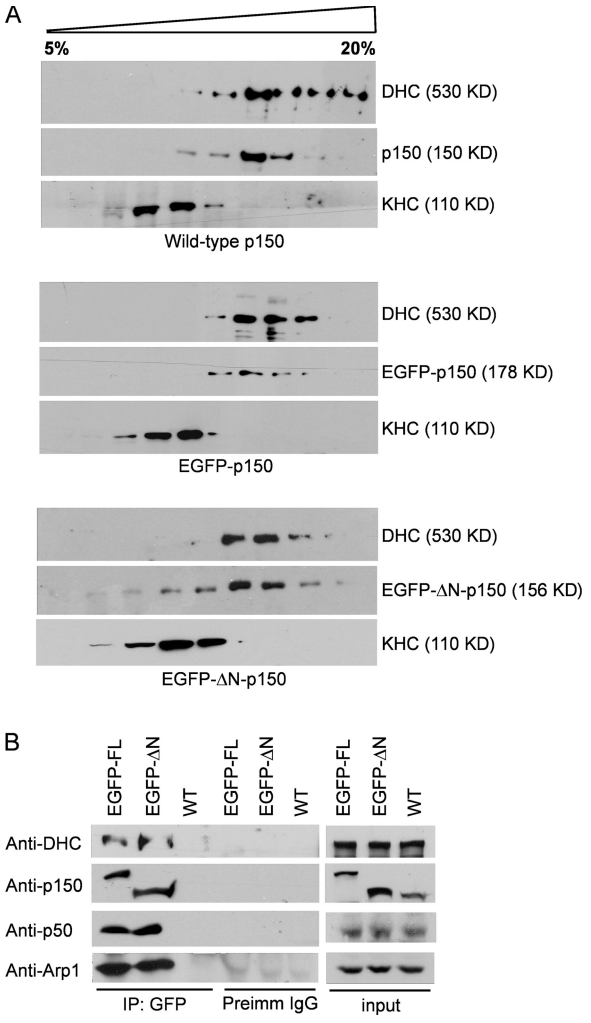

Figure 2. **The truncated form of p150^{glued} and ΔN-p150^{glued} forms the dynactin complex and interacts with cytoplasmic dynein.** (A) Sucrose gradient fractionation of extracts from S2 cells expressing wild-type p150^{glued}, EGFP-p150^{glued}, or EGFP–ΔN-p150^{glued}. EGFP–ΔN-p150^{glued} sediments at the same rate as wild-type p150^{glued} or EGFP-p150^{glued}. KHC is used as a control, which sediments differently from dynein and dynactin. (B) Extracts from cells expressing EGFP-p150^{glued} or EGFP–ΔN-p150^{glued} and untransfected cells were immunoprecipitated with an anti-EGFP antibody or control preimmune IgG. Precipitates were probed using anti-DHC antibody and antibodies against dynactin subunits p150^{glued}, p50, and Arp-1. Note that EGFP antibody but not the control IgG pulls down cytoplasmic dynein and dynactin subunits. Inputs are cell extracts from each sample.

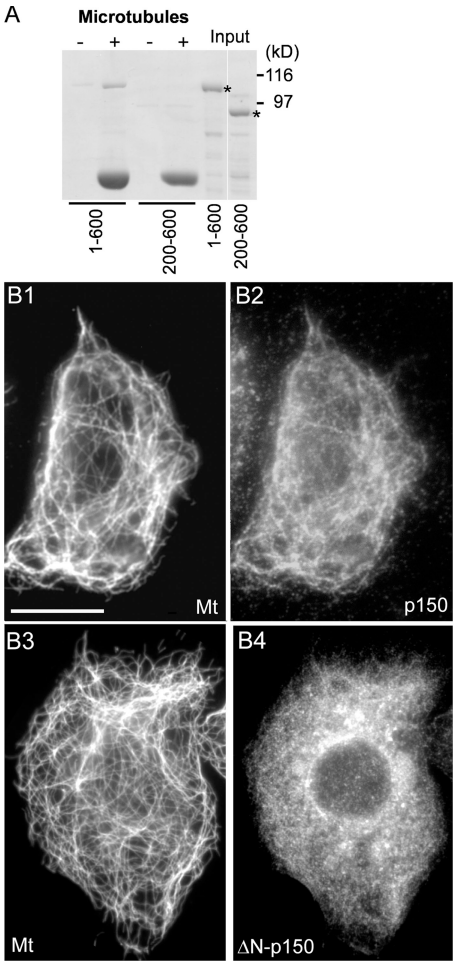

Figure 3. **Truncation of the first 200 amino acid residues of p150^{glued} eliminates microtubule binding.** (A) Recombinant proteins purified and incubated with taxol-stabilized microtubules are centrifuged to pellet with microtubules. Pellets were analyzed by SDS-gel electrophoresis. Inputs are proteins used for the assay, and asterisks mark positions of His$_6$-p150^{glued} (residues 1–600) or His$_6$-p150^{glued} (residues 200–600). (B) EGFP-tagged p150^{glued} (B2) is aligned along microtubules (B1), and EGFP–ΔN-p150^{glued} (B4) shows no microtubule binding. Cells were treated with 3′ UTR RNAi to deplete endogenous p150^{glued}, extracted with detergent, and stained with a monoclonal antibody against α-tubulin and EGFP polyclonal antibody. Bar, 10 μm.

at http://www.jcb.org/cgi/content/full/jcb.200608128/DC1). In some cells, EGFP-p150^{glued} also accumulated at microtubule tips, although this phenotype was not as prominent as in mammalian cells (Video 2; Vaughan et al., 2002). On the other hand, in the cell line expressing EGFP–ΔN-p150^{glued}, microtubule decoration and microtubule tip accumulation were not observed in any focal plane, and p150^{glued} clusters seemed to move randomly in the cytoplasm (Video 3). Thus, we confirm that the first 200 amino acid residues of p150^{glued} contain microtubule-binding activity, and truncation of this domain completely eliminates the ability of p150^{glued} to interact with microtubules.

Dynactin is required for bidirectional cargo transport

To investigate the role of dynactin in the process of microtubule-dependent transport, we used two types of cargo: membranous organelles (peroxisomes, endosomes, and lysosomes) and nonmembranous mRNA–protein (messenger RNP [mRNP]) complexes (*Drosophila* homologue of the fragile X mental retardation protein [dFMRP]). Both EGFP-tagged peroxisomes and EGFP-tagged dFMRP particles have a well-defined morphology and are transported along microtubules by cytoplasmic dynein and conventional kinesin (kinesin-1) as we demonstrated previously (Ling et al., 2004; Kural et al., 2005). To study the movement of cargo along microtubules without the interference of any myosin-dependent components, S2 cells were plated on a concanavalin-A–coated substrate, and actin filaments were depolymerized with cytochalasin D. Under these conditions, cells formed long and thin processes that had a length of 5–20 μm with a diameter of 0.5–1 μm. In addition, as expected, cytochalasin D treatment eliminated ruffling of the lamella and retrograde flow of actin in the lamelloplasm that could contribute to the movement of organelles and could, therefore, interfere with analysis of microtubule-dependent movement. Immunofluorescent staining with an anti–α-tubulin antibody showed that these processes contain microtubule bundles. Microtubules in these bundles have uniform polarity with their plus ends directed toward the tips of the processes (Kural et al., 2005). Both EGFP-tagged peroxisomes (Fig. 4 and Video 4, available at http://www.jcb.org/cgi/content/full/jcb.200608128/DC1) and dFMRP particles (Ling et al., 2004) moved bidirectionally in these processes.

In agreement with our previous results (Ling et al., 2004; Kural et al., 2005), knockdown of either kinesin or cytoplasmic dynein by RNAi abolished the bidirectional motility of peroxisomes along microtubules (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200608128/DC1). In addition, knockdown of either kinesin-1 or dynein by RNAi completely inhibited the motility of lysosomes and endosomes along microtubules (unpublished data). Inhibition of bidirectional movements after the RNAi-induced depletion of one motor is not caused by the depletion of the motor of the opposite polarity because the other motor is still present both in the soluble pool and in the organelle fraction (Fig. S2). These results agreed with a previous study showing the coordination of plus and minus end–directed movements of lipid droplets in *Drosophila* embryos (Gross et al., 2002) as well as a study showing an interdependence of cytoplasmic dynein, the dynactin complex, and kinesin in fast axonal transport in *Drosophila* neurons (Martin et al., 1999).

To study the role of dynactin in cargo transport, we knocked down either p150^{glued} or the p50-dynamitin subunit of the *Drosophila* dynactin complex. Such treatment effectively reduced the bidirectional transport of both types of cargo, demonstrating that dynactin is required not only for the transport of membranous organelles but also for the transport of mRNP particles along microtubules (Fig. 5 A and Table S1, available at http://www.jcb.org/cgi/content/full/jcb.200608128/DC1). RNAi against a mitotic kinesin, Klp61F, was used as a control.

To further confirm the role of dynactin in cargo transport, we overexpressed mRFP fusion proteins with either p50-dynamitin or the first coiled-coil region (amino acid residues 232–583) of p150^{glued}. Both constructs act as dominant-negative inhibitors of dynactin-dependent cellular processes (Burkhardt

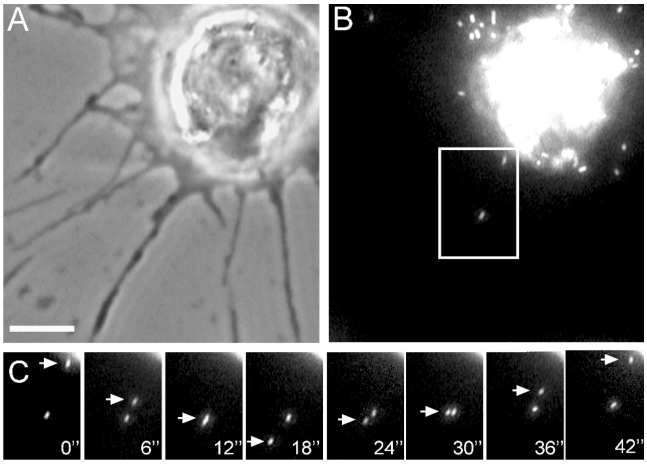

Figure 4. **Morphology and peroxisome distribution in S2 cells spread on the substrate in the presence of cytochalasin D.** (A and B) Phase-contrast (A) and fluorescent (B) images of an S2 cell expressing EGFP-tagged peroxisomes in the presence of cytochalasin D. (C) Frames from a time-lapse video (Video 4, available at http://www.jcb.org/cgi/content/full/jcb.200608128/DC1) show bidirectional movements of peroxisomes. Frames correspond to the boxed area in B. Time in seconds is indicated on each frame. Arrows show a peroxisome moving in the process. Bar, 5 μm.

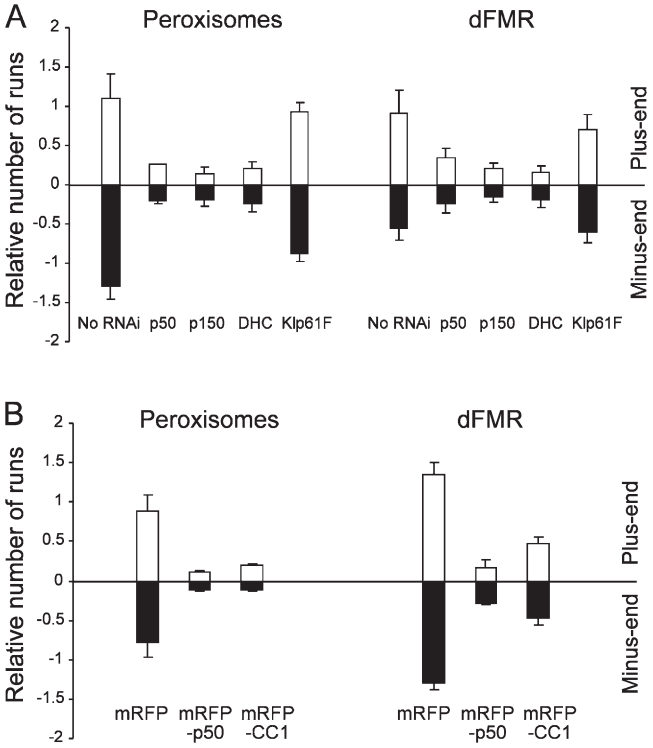

Figure 5. **Dynactin and dynein are required for the bidirectional movement of peroxisomes and dFMRP particles.** (A) The relative number of runs (see Materials and methods) of both peroxisomes and dFMRP particles dramatically dropped after the knockdown of dynactin subunits (p50 or p150^{glued}) or DHC. RNAi against the mitotic kinesin Klp61F was used as a control. (B) The relative number of runs of both peroxisomes and dFMRP particles substantially dropped after the overexpression of dominant-negative inhibitors of dynactin, mRFP-p50, or mRFP-tagged coiled-coil 1 of p150^{glued} (mRFP-CC1). Error bars represent SD.

et al., 1997; Waterman-Storer et al., 1997; Quintyne et al., 1999). As shown in Fig. 5 B, the overexpression of either protein dramatically inhibited the transport of peroxisomes and dFMRP particles. It is worth noting that as in the case of dynactin knockdown, both the plus and minus end movements were blocked by the overexpression of dynactin subunits. The binding of both kinesin and dynein to dynactin could be one potential explanation of the inhibition of bidirectional movement in cells after the knockdown of dynactin subunits or overexpression of dominant-negative constructs. We performed immunoprecipitation assays to test this possibility. An antibody against p150^{glued} pulled down DHC but not KHC from S2 extracts. Similarly, a kinesin antibody did not pull down p150^{glued}, although, in agreement with a previous study (Ligon et al., 2004), it did pull down DHC (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200608128/DC1). Thus, we conclude that dynactin is absolutely required for the bidirectional transport of both membranous (peroxisomes) and nonmembranous (mRNP) cargoes along microtubules, and this result cannot be explained by simultaneous binding of dynactin to the motor proteins of opposite polarity.

Dynactin interaction with microtubules is not required for processive movement of cargo by dynein or kinesin in vivo

To determine the role of the microtubule-binding domain of p150^{glued}, we analyzed the movement of EGFP-tagged peroxisomes and dFMRP particles in S2 cells expressing either mRFP-p150^{glued} or mRFP–ΔN-p150^{glued}. As mentioned above (see Generation of cell lines and RNAi procedure), we treated cells with RNAi against the 3′ UTR of p150^{glued} mRNA to deplete endogenous p150^{glued}. The observation of cargo movement in these cells indicated that the replacement of wild-type p150^{glued} with either mRFP-p150^{glued} or mRFP–ΔN-p150^{glued} had no effect on peroxisome or dFMRP particle transport. Similar to the wild-type cells, cells expressing ΔN-p150^{glued} showed multiple EGFP-labeled cargo moving bidirectionally within cellular processes (Videos 5 and 6, available at http://www.jcb.org/cgi/content/full/jcb.200608128/DC1), whereas cells depleted of p150^{glued} demonstrated no movement (not depicted).

Quantitative analysis demonstrated that removal of the microtubule-binding domain of p150^{glued} did not affect the long-range movement of peroxisomes or dFMRP particles along microtubules. Unlike the total depletion of p150^{glued}, replacement with mRFP–ΔN-p150^{glued} had no effect on the mean run length of either peroxisomes or dFMRP particles (Fig. 6 A). We also compared two other parameters of movement: the relative number of runs and the mean velocity of runs (Fig. 6 B, Fig. S4 A, and Table S1, available at http://www.jcb.org/cgi/content/full/jcb.200608128/DC1). The relative number of runs was determined as the number of runs longer than a threshold value normalized to the number of analyzed organelles (see Materials and methods). This parameter should be most dramatically affected if the processivity of organelle movement was changed. In agreement with the mean run length data, the number of runs and velocity of peroxisome and dFMRP movements in both directions were not affected by removal of the microtubule-binding domain of p150^{glued}.

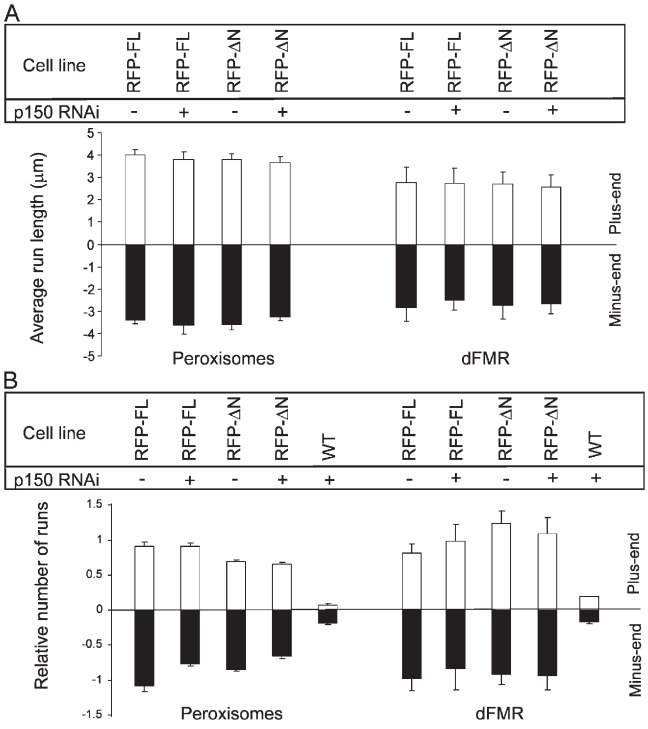

Figure 6. **Deletion of the microtubule-binding domain of p150^{glued} has no effect on the mean run length or relative number of runs of both peroxisomes and dFMRP particles.** (A) Mean run length; (B) relative number of runs. Note that the relative number of runs in cells having no p150^{glued} expression dropped more than fivefold compared with the cells expressing mRFP-p150^{glued} or mRFP–ΔN-p150^{glued}. The mean run length is calculated from the trajectory length of moving particles in the process without stopping or changing directions. The total number of particles analyzed for each treatment group in this experiment was 83, 147, 256, 268, and 113 for peroxisomes and 172, 142, 170, 337, and 111 for dFMR (numbers correspond to bars on the chart from left to right). Error bars represent SD.

To exclude the possibility that the aforementioned results are specific to these two particular kinds of cargo, we measured the effects of removal of the microtubule-binding domain of p150^{glued} on the movement of endosomes and lysosomes. Endosomes were labeled by the incubation of cells with Texas red–conjugated dextran, and lysosomes were labeled with Lysotracker. We found that similar to peroxisomes and dFMR, the movement of lysosomes and endosomes in cytochalasin-treated cells was not affected by truncation of the microtubule-binding domain (Fig. S4 B and Table S1). We also examined the distribution of organelles in cells not treated with cytochalasin D. We could not see any effects of truncation of the microtubule-binding domain on the steady state distribution of any organelles studied here (Fig. S4 C), suggesting that even in the presence of actin, organelle movement is not affected by truncation of the microtubule-binding domain. Collectively, these results demonstrate that the presence of the microtubule-binding domain of p150^{glued} is not important for the movement of at least four different types of cargo along microtubules in S2 cells.

Previously, we showed that both kinesin and cytoplasmic dynein move peroxisomes along microtubules in discrete 8-nm steps in vivo that correspond to the step size of both motors in vitro (Kural et al., 2005). Similar discrete steps were clearly seen in traces of peroxisomes in cells expressing mRFP-p150^{glued} or mRFP–ΔN-p150^{glued} and depleted of endogenous p150^{glued}. As shown in Fig. 7, the mean step sizes for kinesin and cytoplasmic dynein in cells expressing mRFP–ΔN-p150^{glued} are 7.7 ± 1.8 nm and 8.9 ± 1.4 nm, respectively. These numbers are not substantially different from the mean step sizes of kinesin and dynein in cells expressing mRFP-p150^{glued} (9.6 ± 3.1 nm and 8.8 ± 2.4 nm, respectively) or in wild-type cells as previously shown (Kural et al., 2005). We conclude that deletion of the microtubule-binding domain of p150^{glued} does not affect the characteristics of the mechanochemical cycle of molecular motors in vivo.

Dynactin binding to microtubules suppresses the generation of multipolar spindles and free MTOCs

Drosophila cytoplasmic dynein and dynactin play critical roles during cell division that include spindle assembly and elongation, anaphase chromosome movements, and removal of spindle checkpoint components from attached kinetochores (Robinson et al., 1999; Sharp et al., 2000a,b; Wojcik et al., 2001; Buffin et al., 2005; Morales-Mulia and Scholey, 2005). To examine the mitotic contribution of the microtubule-binding domain of p150^{glued}, S2 cells expressing EGFP-p150^{glued} or EGFP–ΔN-p150^{glued} were treated with either control or double-stranded RNA from the 3′ UTR of p150^{glued} and immunostained for both microtubules and the mitosis-specific phosphorylated histone H3. Depletion of endogenous p150^{glued} in cells expressing EGFP–ΔN-p150^{glued} elevated the mitotic index threefold and increased the frequency of prometaphase stage figures as compared with control RNAi–treated cells. A similar prometaphase-like arrest was also described for cultured mammalian cells overexpressing the dynactin inhibitor p50/dynamitin (Echeverri et al., 1996). Unexpectedly, we also observed a substantial increase in multipolar spindles in cells expressing EGFP–ΔN-p150^{glued} (Fig. 8, A and B2), a phenotype not previously described for dynactin inhibition. We attribute multipolar spindle formation to the failure to properly coalesce MTOCs during prometaphase, a mechanism that may depend on a p150^{glued}–microtubule interaction.

A previous study found that 39% of untreated prophase S2 cells contain four or more γ-tubulin–staining MTOCs, and live imaging of S2 cells expressing GFP-tubulin revealed a clustering and fusion mechanism to eliminate extra MTOCs after nuclear envelope breakdown (Goshima and Vale, 2003). Consequently, failure to cluster and fuse extra MTOCs resulted in multipolar spindle formation. Furthermore, Quintyne et al. (2005) identified cytoplasmic dynein as a critical component of a centrosome-clustering mechanism present in human tissue culture cells that, when mislocalized in certain tumor cells, results in multipolar spindle formation. Consistent with this hypothesis, we found that cells expressing EGFP–ΔN-p150^{glued} exhibited a dramatic increase in the number of free MTOCs surrounding mitotic spindles (Fig. 8, A and B3). Thus, the microtubule-binding domain of p150^{glued} is required to suppress multipolar spindle formation, probably by coalescing extra MTOCs that are frequent in many early mitotic S2 cells.

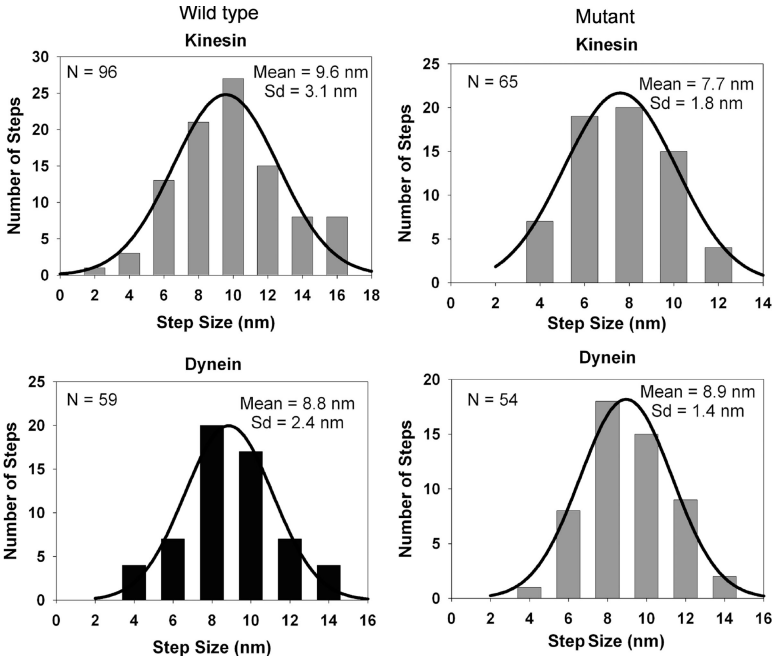

Figure 7. **Step sizes of cytoplasmic dynein– or kinesin-transporting peroxisomes are not altered by truncation of the microtubule-binding domain of p150^{glued}.** Histograms of the distribution of the individual steps of kinesin and dynein show that the mean step sizes are ~8 nm in both wild-type (mRFP-p150^{glued}) and mutant (mRFP–ΔN-p150^{glued}) cells.

Discussion

Adaptors between motors and cargo are a diverse group of cellular proteins, but the most universal of them is the dynactin complex. An unusual property of this complex is its ability to bind microtubules independently of motor proteins. In this study, we directly addressed the functional significance of the microtubule binding of dynactin by replacing endogenous p150^{glued} with a p150^{glued} mutant lacking residues 1–200, which was thus unable to bind microtubules (ΔN-p150^{glued}). We selected stable cell lines expressing mRFP-tagged p150^{glued} or ΔN-p150^{glued} and depleted endogenous p150^{glued} by using RNAi. This approach allowed us to study the contribution of the microtubule-binding domain of p150^{glued} to cargo transport and the organization of mitotic microtubule arrays. We demonstrated that the movement of two types of cargo, membranous organelles (peroxisomes, endosomes, and lysosomes) and mRNP complexes (dFMRP particles), is absolutely dependent on the dynactin complex, but, to our surprise, ΔN-p150^{glued}, which lacks the ability to bind microtubules, was fully functional in supporting microtubule-dependent transport in vivo. All of the measurements were performed in the cells treated with cytochalasin D to eliminate actin-based motility.

It should be stressed that this study showed for the first time that dynactin is directly involved in RNA transport in somatic cells. Although a previous study suggested that dynactin is required for the proper localization of morphogen RNA in *Drosophila* oocytes (Januschke et al., 2002), direct evidence has been lacking. On the other hand, we observed a profound effect of truncation of the microtubule-binding domain on mitotic spindle structure, including the generation of multipolar spindles and free MTOCs. Although dynactin plays an important role in organizing the interphase radial microtubule arrays at the centrosome in mammalian cultured cells (Quintyne et al., 1999; Askham et al., 2002; Quintyne and Schroer, 2002), we observed no change in the pattern of interphase microtubules in S2 cells expressing ΔN-p150^{glued}. This was expected given that S2 cells lack functional centrosomes capable of nucleating and organizing microtubules during interphase. Instead, microtubules nucleate

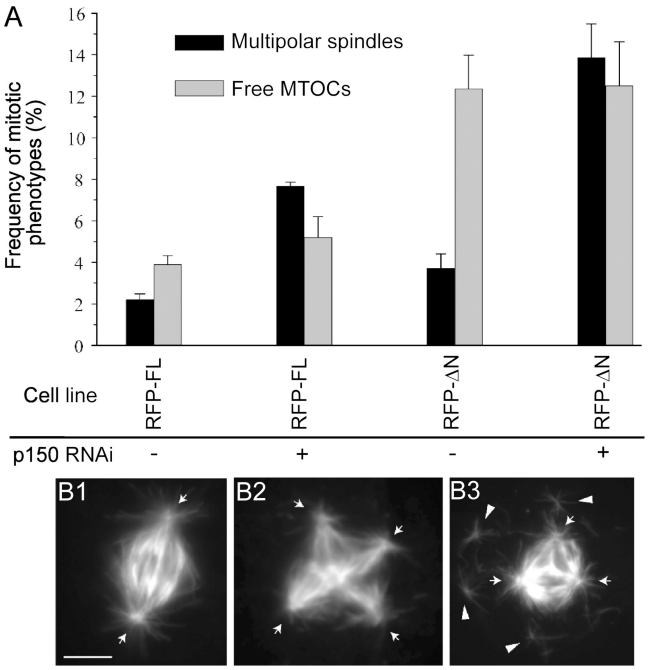

Figure 8. **Replacement of p150^{glued} with EGFP–ΔN-p150^{glued} results in the accumulation of multipolar spindles and nonspindle-associated MTOCs during cell division.** (A) Percentage of multipolar spindles and free MTOCs present in a mean of 200 mitotic cells per RNAi treatment. Error bars represent SD. (B) Mitotic cells stained for microtubules. (B1) Normal metaphase in the control cell. (B2 and B3) Multipolar spindles (B2) and free MTOCs (B3) in cells expressing EGFP–ΔN-p150^{glued}. Free MTOCs are labeled with arrowheads, and spindle poles are marked with arrows. Bar, 5 μm.

at random in the cytoplasm and do not form a focused radial array (unpublished data).

It is worth noting that one of the p150^{glued} isoforms in the mammalian brain is an alternatively spliced version called p135, which naturally lacks the microtubule-binding motif (Tokito et al., 1996). As neurons are terminally differentiated cells that do not divide or contain radial arrays of interphase microtubules, they do not need dynactin activity to facilitate microtubule organization. On the other hand, the results presented here suggest that this alternatively spliced isoform is as effective as full-length p150^{glued} in supporting transport.

Examination of the database shows that there is a second *p150* gene in *Drosophila* (gi|23093121|gb|AAF49148.2|; Goldstein and Gunawardena, 2000). We performed additional experiments addressing whether the existence of this second gene rescues and compensates for the loss of the normal p150^{glued} protein. RNAi against this gene did not change organelle movement either in the wild-type or mutant background. On the other hand, RNAi against conventional p150^{glued} alone completely blocked movement. We assume that this second gene is not important for organelle transport or is not even expressed in S2 cells.

It has been reported recently that a G59S substitution in the microtubule-binding domain of p150^{glued} results in decreased microtubule binding and enhanced dynein and dynactin aggregation (Levy et al., 2006). It is known that this substitution results in a slowly progressing motor neuron disease in humans (Puls et al., 2003), suggesting the impairment of dynactin functions. Although this mutation could directly affect organelle transport by altering the microtubule-binding affinity of the dynactin complex, it is also possible that it could inactivate dynactin by forming aggregates that recruit both wild-type and mutant dynactin, resulting in a decrease in the level of functional dynein–dynactin complex available for cargo transport.

Three possible functions have been proposed for the microtubule-binding domain of p150^{glued}. First, King et al. (2000) demonstrated in vitro that the dynactin complex increases the processive movement of beads coated with cytoplasmic dynein. This idea is further supported by two recent studies (Culver-Hanlon et al., 2006; Kobayashi et al., 2006) showing that p150^{glued} is capable of one-dimensional diffusion along microtubules and, thus, maintains contact with microtubules. Therefore, enhanced processivity in vitro can be achieved by the p150^{glued}–microtubule interaction. In contrast, the results shown here indicate that the effect of dynactin on motor processivity is sufficiently minor, and it is not detected by tracking cargoes in vivo even with the 1-nm, 1-ms accuracy of the FIONA technique (Yildiz and Selvin, 2005). There are several potential explanations of an apparent discrepancy between our results and the previous in vitro studies (King and Schroer, 2000; King et al., 2003). First, it is not known whether the four types of cargo studied here are transported by single or multiple motors. If cargo were transported by more than one motor, dynactin would not contribute to the processivity. Furthermore, several redundant mechanisms may be involved in the regulation of dynein processivity in cells, and, thus, the effects of truncation of the microtubule-binding domain might not be immediately obvious if other putative mechanisms are in action. Second, previous studies (King and Schroer, 2000; Culver-Hanlon et al., 2006) were performed in vitro not with the native dynein–dynactin complex but with two proteins bound separately to the surface of carboxylated beads. It is unclear whether all of the properties of the dynein–dynactin complex on the surface of organelles can be faithfully recapitulated by the binding of purified proteins with highly charged beads. Finally, in vitro assays were performed under no load condition, and it is possible that the load applied to organelles in the cytoplasm affects the processivity of transport.

The second potential role of the microtubule-binding domain of p150^{glued} was proposed by Vaughan et al. (2002; Vaughan, 2005a). These authors suggested that p150^{glued} tethering to the plus ends of growing microtubules facilitates the loading of retrograde cargo on the plus ends of microtubules. This function would require the interaction of dynactin with microtubules. Obviously, the truncated form of p150^{glued}, ΔN-p150^{glued}, could not load cargo onto microtubules. However, at least in the case of the two types of cargo we examined here, such a search and capture mechanism is not a major contributor to the retrograde transport by dynein because the truncation of microtubule binding from dynactin had no effect on cargo transport. In agreement with these results, Watson and Stephens (2006) showed that depletion of either EB1 or CLIP-170 resulted in a loss of p150^{glued} from microtubule plus ends but had no effect on the trafficking of membrane organelles. It is likely that a combination of microtubule dynamics and a Brownian motion of cargo is sufficient for making initial contact and loading of cargo onto microtubules. Thus, microtubule plus end targeting of p150^{glued} is not required for cargo loading. Still, it would be interesting to examine the effect of ΔN-p150^{glued} on cargo transport in the cellular regions containing single microtubules instead of cytochalasin D–induced microtubule bundles to detect the kinetic advantages of search and capture if they existed.

Finally, the microtubule-binding domain of p150^{glued} interacts directly with the C terminus of CLIP-170 (Lansbergen et al., 2004) or EB1 (Askham et al., 2002; Hayashi et al., 2005). Dynactin has potential roles in regulating microtubule dynamics at the plus end complex and is required for microtubule anchoring and focusing in a cell cycle–dependent manner. It is likely that p150^{glued} links proteins at the cell cortex with cytoplasmic dynein or tip-binding proteins to pull and focus microtubules, thus preventing the formation of multipolar spindles or free MTOCs during cell division. We observed a dramatic effect of truncation of the microtubule-binding domain on mitotic spindle structure, stressing the essential role of the N-terminal microtubule-binding domain of dynactin in microtubule organization. This supports the role of centrosomal dynactin for the microtubule anchoring function proposed by Quintyne et al. (1999). Collectively, our data demonstrate that the microtubule-binding domain of p150^{glued} has a substantial role in spindle microtubule orientation and focusing but is not required for long-range movement of cargoes along microtubules by motor proteins in vivo.

Materials and methods

Molecular cloning

The cDNA for *Drosophila* p150^{glued} (clone AY118377; Open Biosystems) and the ΔN-p150^{glued} construct encoding amino acid residues 201–1,280 were amplified and subcloned into the pAc5.1/V5-HisA vector (Invitrogen). The EGFP or mRFP sequence was introduced at the N terminus of p150^{glued}. To make His_6-tagged p150^{glued}, the sequence of p150^{glued} encoding residues 1–600 or 200–600 was subcloned into pET28 (a+; Novagen/EMD Biosciences). The first coiled-coil domain (CC1; amino acids 232–583) of p150^{glued} or full-length p50/dynamitin was fused to mRFP to create the dominant-negative constructs mRFP-CC1 and mRFP-p50 in the pMT5.1/V5-HisA vector.

Cell culture

To select stable cell lines expressing EGFP-SKL (peroxisome targeting signal) and mRFP-p150^{glued} or mRFP–ΔN-p150^{glued}, S2 cells were cotransfected with three plasmids: pGG101 encoding EGFP-SKL (a gift from G. Goshima, University of California, San Francisco, San Francisco, CA), pCoHygro (Invitrogen) as a selection plasmid, and mRFP-p150^{glued} or mRFP–ΔN-p150^{glued} at a 20:1:20 molar ratio. Transfection was performed using Cellfectin reagent (Invitrogen). 40 h after transfection, 300 μg/ml hygromycin was added for selection. Selection was performed for 4–5 wk, and protein expression was confirmed by fluorescence microscopy and immunoblotting. Lysosomes in S2 cells were labeled by staining with 100 nM Lysotracker red DND-99 (Invitrogen) for 10 min. For endosome labeling, S2 cells in suspension were incubated with 1 mg/ml Texas red dextran (Invitrogen) for 6 h.

Double-stranded RNAi

RNAi treatment was performed as described previously (Ling et al., 2004). Templates for in vitro transcription were generated by using the primers 5′-TAATACGACTCACTATAGGGGTATCGTGGCAATGGAATCG-3′ and 5′-TAATACGACTCACTATAGGGGAGTTATACAACATCAGCAA-3′ to amplify the 500 bp from the 3′ UTR of p150^{glued}, and the primers 5′-TAATACGACTC-ACTATAGGGACCACCTGCAAAGCGATATCG-3′ and 5′-TAATACGACTCACTA-TAGGGTTGCAGATACTCCGTCAGGAT-3′ were used to amplify the 550-bp segment from the C terminus of the *p150^{glued}* gene.

Antibodies

The recombinant His_6-tagged p150^{glued} fusion proteins containing residues 1–190 or 1,073–1,280 were expressed in *Escherichia coli* and purified by Talon affinity chromatography. Rabbit immunization was performed by Proteintech Group, Inc. For Western analysis, a 1:5,000 dilution of antiserum was used. An antibody against DHC was provided by J. Scholey (University of California, Davis, Davis, CA), and HD antibody against KHC was provided by A. Minin (Institute of Protein Research, Russian Academy of Sciences, Moscow, Russia). An antibody against p50 was a gift from R. Warrior (University of California, Irvine, Irvine, CA; Duncan and Warrior, 2002), and Arp1 antibody was provided by L. Goldstein (University of California, San Diego, La Jolla, CA). SUK4 and 9E10.2 (anti-KHC and anti-myc antibody, respectively) were obtained from the Developmental Studies Hybridoma Bank.

Immunofluorescent staining

Cells were incubated in extraction buffer containing 1% Triton X-100 in PBS for 2 min and fixed with 1% glutaraldehyde or cold methanol for 10 min. For microtubule or EGFP staining, we used monoclonal antibody DM1-α against α-tubulin (1:2,000) and polyclonal affinity-purified EGFP antibody (1:200), respectively. For analysis of the mitotic phenotype, cells were immunostained for both microtubules and mitosis-specific phosphorylated histone H3.

Immunoprecipitation

Approximately 10^8 cells were used for the immunoprecipitation assay. Cell pellets were resuspended at a ratio of 1:2 (wt/vol) in a homogenization buffer (50 mM Tris, pH 7.5, 50 mM KCl, 0.1 mM EDTA, 2 mM $MgCl_2$, 1% NP-40, 5% glycerol, 1 mM DTT, 1 mM PMSF, and 10 μg/ml each of chymostatin, leupeptin, and pepstatin) and homogenized by using a 25-gauge syringe needle. Cell extracts were centrifuged at 15,000 *g* for 10 min and then at 200,000 *g* for 15 min. The resulting supernatants were incubated with antibodies (EGFP or preimmune rabbit IgG) prebound to protein A–Sepharose beads (GE Healthcare) for 2 h at 4°C. The beads were washed, and proteins were eluted in SDS sample buffer and analyzed by Western blotting.

Microscopy and image analysis

Images of live cells were acquired as described previously (Ling et al., 2004) using a microscope system (U2000 Perfect Focus; Nikon). A 100-W halogen bulb was used for fluorescence excitation to minimize photobleaching and phototoxicity. Images were captured every 1 s for 2 min for EGFP-tagged peroxisomes, endosomes, and lysosomes and every 2 s for 2 min for EGFP-tagged dFMRP. The movement of particles was analyzed by using the automatic tracking software Diatrack (version 3.01; Semasopht). The threshold speed was 0.2 μm/s for peroxisomes, endosomes, and lysosomes or 0.15 μm/s for dFMRP particles, and movements slower than this threshold were excluded from the calculations. We measured all runs longer than 2 μm for peroxisomes, longer than 1.6 μm for endosomes and lysosomes, and longer than 1 μm for dFMRP particles. The number of runs above the threshold was divided by the mean number of particles in the analyzed areas of the image. This value was defined as the relative number of runs. At least three independent experiments were analyzed for each condition, and five to six cells in each experiment were randomly chosen for recording and analysis.

Sucrose density gradient centrifugation

Approximately 3×10^7 cells were pelleted, and cell extract was prepared as described in the Immunoprecipitation section. ~500 μl of the clarified supernatant was layered on top of 12 ml of 5–20% linear sucrose density gradient prepared in the homogenization buffer without NP-40. After centrifugation at 150,000 *g* for 18 h in a SW40 rotor (Beckman Coulter), 0.5-ml fractions were collected and analyzed by Western blotting using antibodies to EGFP, p150^{glued}, DHC, or KHC.

Microtubule pelleting assay

Microtubules were prepared by polymerization of bovine brain tubulin in the presence of 1 mM GTP and 20 μM taxol at 37°C for 1 h. The polymerized microtubules were mixed with recombinant proteins and layered on top of a 30% glycerol cushion in BRB80 buffer (80 mM Pipes, pH 6.9, 1 mM EGTA, and 1 mM $MgCl_2$) with 10 μM taxol. Microtubules were pelleted by centrifugation at 150,000 *g* for 40 min in a SW55 rotor (Beckman Coulter). The pellets were washed and resuspended in 30 μl SDS sample buffer.

Online supplemental material

Fig. S1 shows that the depletion of cytoplasmic dynein or kinesin stops peroxisome movement. Fig. S2 shows that the depletion of one motor (kinesin or dynein) does not deplete the other motor of opposite polarity. Fig. S3 shows that kinesin can interact with dynein but not with dynactin. Fig. S4 A shows that truncation of the microtubule-binding domain of p150^{glued} has no effect on the mean velocity of peroxisome or dFMR particles. Fig. S4 B shows that truncation of the microtubule-binding domain of p150^{glued} has no effect on mean run length, relative number of runs, and mean velocity of endosomes or lysosomes. Fig. S4 C demonstrates that the steady-state distribution of organelles in S2 cells not treated with cytochalasin D is not affected by the expression of mRFP-p150^{glued} or mRFP–ΔN-p150 ^{glued}. Table S1 shows absolute and relative numbers of long runs in cells treated with RNAi against motors or dynactin components, cells overexpressing dynactin subunits, and cells expressing EGFP-p150^{glued} or EGFP–ΔN-p150^{glued}. Videos 1 and 2 demonstrate that EGFP-p150^{glued} decorates tips and the length of microtubules. Video 3 demonstrates that EGFP–ΔN-p150^{glued} is distributed randomly in the cytoplasm. Videos 4–6 show bidirectional movements of peroxisomes in the processes of S2 cells expressing endogenous p150^{glued}, mRFP-p150^{glued}, and mRFP–ΔN-p150^{glued}, respectively. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200608128/DC1.

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