Dynactin Is Required for Coordinated Bidirectional Motility, but Not for Dynein Membrane Attachment^D ^V

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Transport of cellular and neuronal vesicles, organelles, and other particles along microtubules requires the molecular motor protein dynein (Mallik and Gross, 2004). Critical to dynein function is dynactin, a multiprotein complex commonly thought to be required for dynein attachment to membrane compartments (Karki and Holzbaur, 1999). Recent work also has found that mutations in dynactin can cause the human motor neuron disease amyotrophic lateral sclerosis (Puls *et al.*, 2003). Thus, it is essential to understand the *in vivo* function of dynactin. To test directly and rigorously the hypothesis that dynactin is required to attach dynein to membranes, we used both a *Drosophila* mutant and RNA interference to generate organisms and cells lacking the critical dynactin subunit, actin-related protein 1. Contrary to expectation, we found that apparently normal amounts of dynein associate with membrane compartments in the absence of a fully assembled dynactin complex. In addition, anterograde and retrograde organelle movement in dynactin deficient axons was completely disrupted, resulting in substantial changes in vesicle kinematic properties. Although effects on retrograde transport are predicted by the proposed function of dynactin as a regulator of dynein processivity, the additional effects we observed on anterograde transport also suggest potential roles for dynactin in mediating kinesin-driven transport and in coordinating the activity of opposing motors (King and Schroer, 2000).

INTRODUCTION

Dynein is the major molecular motor protein responsible for a variety of microtubule-based minus-end-directed movements of vesicles and organelles as well as several steps in mitosis (Karki and Holzbaur, 1999; Mallik and Gross, 2004; Pilling *et al.*, 2006). A key gap in our understanding of dynein function is how this motor protein interacts with membrane compartments. One candidate factor proposed to link dynein to membrane compartments is a multiprotein complex called dynactin (for review, see Schroer, 2004). Although the original work on dynactin suggested that highly purified dynein could mediate vesicle attachment to microtubules in the absence of dynactin, a more recent, and relatively small number of *in vitro* experiments have led to

the generally accepted model that the attachment of dynein to membrane vesicles requires dynactin (Waterman-Storer *et al.*, 1997; Karki and Holzbaur, 1999; Muresan *et al.*, 2001). An alternative model suggests that dynein light and intermediate chain (IC) subunits may link dynein to other membrane-associated proteins independently of dynactin (Tai *et al.*, 1999; Tynan *et al.*, 2000; Yano *et al.*, 2001). In this competing view, dynactin plays a role in regulating or coordinating dynein functions such as processivity (King and Schroer, 2000). Understanding the role of dynactin in dynein function has recently become more important with the realization that these proteins may be targets in human neurodegenerative diseases (Hafezparast *et al.*, 2003; Puls *et al.*, 2003). Yet, in spite of the importance of this issue, definitive evidence on the *in vivo* role of dynactin in dynein attachment to membranes does not exist, and no direct experiment testing whether dynactin is required for linking dynein to membranes *in vivo* has been reported.

The actin-related protein Arp1 is the most abundant subunit of the dynactin multiprotein complex. This 45-kDa protein forms a filament composed of eight to 13 monomers, which is capped by the p37 and p32 capping proteins on one end (also known as CapZ) and the p62 subunit of dynactin on the other end. These proteins in addition to some smaller subunits, such as p25, p27, and Arp11 form a scaffold upon which p150^{Glued} binds to the Arp1 filament through an interaction mediated by another protein called dynamitin (for review, see Schroer, 2004). Dynein interacts with the dynactin complex via binding of the dynein IC to the

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Abbreviations used: Arp1, actin-related protein 1; DHC, dynein heavy chain; KHC, kinesin heavy chain; p50, dynamitin; PNS, post-nuclear supernatant; HSP, high-speed pellet; HSS, high-speed supernatant.

p150^{Glued} subunit of dynactin (Karki and Holzbaur, 1995; Vaughan and Vallee, 1995). Disruption of dynactin functions leads to phenotypes that closely mimic those observed in dynein heavy chain (DHC) and dynein light chain mutants and in antibody inhibition studies (Karki and Holzbaur, 1999). These data suggest that dynein and dynactin work together to carry out dynein functions, although they leave unresolved the question of how dynactin function is required for dynein activity. To elucidate the function of dynactin and to test directly whether dynactin is required to attach dynein to membranes *in vivo*, we analyzed *arp1* mutations and *arp1* RNA interference in *Drosophila*.

MATERIALS AND METHODS

Identification and Mapping of *arp1* Mutants

Homozygous lethal ethyl methanesulfonate (EMS) mutant lines were obtained from the laboratory of Dr. Charles Zuker (University of California, San Diego, La Jolla, CA). The mutant third instars were examined for sluggish crawling and tail flip phenotypes. The *arp1* mutants (previously called *gridlock*) were mapped to cytological position 87C on the third chromosome of *Drosophila* by deficiency mapping and meiotic recombination. This region includes the *arp1* gene. Genomic *arp1* DNA was sequenced from homozygous third instars.

Immunostaining

Larval segmental nerve immunostaining was performed as described previously (Hurd and Saxton, 1996) and observed using a Bio-Rad MRC1024 confocal microscope (Gindhart *et al.*, 1998).

Antibodies

Anti-dynein heavy chain antibody (P1H4) (a generous gift from Tom Hays, Department of Genetics and Cell Biology, University of Minnesota, St. Paul, MN 55108), anti-p150^{Glued} (a generous gift from Erika Holzbaur, School of Medicine, University of Pennsylvania, Philadelphia, PA 19104), anti-dynamitin (p50) (gift from Jason Duncan, Department of Cellular and Molecular Medicine, University of California, San Diego, 9500 Gilman Dr., LBR 411, La Jolla, CA 92093), anti-syntaxin (8C3) (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), anti-kinesin heavy chain (KHC) (Cytoskeleton, Denver, CO), anti-Rab8 and cyt c (BD Biosciences Transduction Laboratories, Lexington, KY), anti-tubulin and anti-actin (Sigma-Aldrich, St. Louis, MO), anti-dynein intermediate chain (Chemicon International, Temecula, CA), and anti-d120 (Calbiochem, San Diego, CA) were used as described.

Arp1 Fusion Protein and Antibody Preparation

Seven *Arp1* expressed sequence tag clones were found in the Berkeley *Drosophila* Genome Project database. Only one contained a complete *Arp1* cDNA (clone GH17B). This clone was purchased from Research Genetics (Huntsville, AL), fully sequenced, and then subcloned into the pET-23b vector (Novagen, Madison, WI), which includes a 6x His tag at the C terminus. The expressed fusion protein was purified using nickel-nitrilotriacetic acid agarose resins (QIAGEN, Valencia, CA), electrophoretically isolated, and used to raise antisera in rabbits (Lampire Biological Laboratories, Pipersville, PA). The specificity of the *Drosophila* *Arp1* antibody was confirmed by Western blotting: a 1:10,000 dilution of the serum detected 5 ng of the recombinant protein and also detected *Arp1* in 10 μ g of total protein from larval brain extracts. We concluded that the *Arp1* antibody cross-reacts with actin, based on the observation that reduction of *Arp1* reactivity after *arp1* double-stranded RNA (dsRNA) treatment is not obvious in the high-speed supernatant where actin is abundant, whereas reduction of *Arp1* in the high-speed pellet fraction where no actin can be detected is obvious (Figure 2A). In addition, sucrose density sedimentation analyses of high-speed supernatants showed a peak at ~8S with sedimentation behavior that is identical to actin in both green fluorescent protein (GFP) dsRNA and *arp1* dsRNA-treated S2 cells (Figure 2, C and D). This peak is not observed in the high-speed pellet fraction where no actin can be detected (Figure 2, E and F).

S2 Cell Culture

S2 cells were grown and maintained in Schneider's *Drosophila* medium (Invitrogen, Carlsbad, CA)/10% fetal bovine serum at room temperature. dsRNA was generated using the Megascript RNA interference (RNAi) kit (Ambion, Austin, TX) from a 500-base pair polymerase chain reaction (PCR) product by using primers that contain the T7 RNP sequence on the end (Eaton *et al.*, 2002). The dsRNA (25 μ g) was added to 2×10^6 cells in a six-well plate in 1 ml of serum-free media. After 30 min, 3 ml of complete medium was added to each well. Cells were harvested for analysis after 5 d. For reverse

transcriptase-quantitative PCR (RT-qPCR), total RNA from S2 cells was prepared using the RNeasy kit (QIAGEN). The RNA was subsequently treated with DNaseI by using the DNA-free kit (Ambion). First-strand cDNA was generated using SuperScript First-Strand Synthesis system (Invitrogen) for RT-PCR and quantitative PCR was performed in an Mx3000P cycler (Stratagene, La Jolla, CA) by using Brilliant SYBR Green QPCR Master Mix. The percentage reduction of *arp1* mRNA was determined by normalizing to a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control. Primers used for *arp1* were forward, tcgaactgaagaacactcg and reverse, ctgtcctctcctcg-tattc and for GAPDH were forward, aattaaggccaaggttcagga and reverse, accaagatcagcttcacga.

Sucrose Density Gradient

For sucrose density gradient analysis of *Drosophila* larval brains, 250 *arp1* larval brains and 80 wild-type brains were dissected and homogenized in PMEG buffer [0.1 M piperazine-*N,N'*-bis(2-ethanesulfonic acid), pH 6.9, 5 mM EGTA, 0.9M glycerol, 5 mM MgSO₄, 0.1 mM EDTA, 0.5 mM dithiothreitol (DTT), and protease inhibitors] (Hays *et al.*, 1994). The high-speed supernatant was prepared by centrifugation at 50,000 rpm for 40 min in a TLA100.3 rotor (Beckman Coulter, Fullerton, CA), and then overlaid on a continuous 5–20% sucrose gradient and centrifuged at 35,000 rpm for 16 h in a SW41 rotor (Beckman Coulter). For sucrose density gradient analysis of S2 cells, *arp1* dsRNA-treated cells and GFP dsRNA-treated cells were homogenized in buffer A (50 mM Tris, 150 mM NaCl, pH 7.4, and 0.5 mM EDTA) plus protease inhibitors, centrifuged sequentially 10 min at $1000 \times g$, 10 min at $10,000 \times g$, and 50 min at $100,000 \times g$. The high-speed supernatant (HSS) was collected and the high-speed pellet (HSP) was resuspended in buffer A + 1% Triton X-100. HSS and HSP were overlaid on a continuous 5–20% sucrose gradient prepared in buffer A and centrifuged at 35,000 rpm for 16 h in a SW41 rotor. Then, 0.5-ml fractions were collected, and total proteins were precipitated and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and Western blot. Gradients loaded with the markers carbonic anhydrase, bovine serum albumin, alcohol dehydrogenase, β -amylase, catalase, apoferritin, and thyroglobulin were run in parallel as standards to determine 2.8S, 4.2S, 7.5S, 9S, 11S, 17S and 19S, respectively.

Membrane Flotation Assay

Third instars were individually hand dissected, and ~250 brains were collected into dissection buffer (2X stock contains 128 mM NaCl, 4 mM CaCl₂, 4 mM MgCl₂, 2 mM KCl, 5 mM HEPES, and 36 mM sucrose, pH 7.2). The brains were homogenized in acetate buffer (10 mM HEPES, pH 7.4, 100 mM K-acetate, 150 mM sucrose, 5 mM EGTA, 3 mM Mg-acetate, 1 mM DTT, and protease inhibitors). Debris was removed by centrifugation at $1000 \times g$ for 7 min, and the resulting postnuclear supernatant (PNS) was brought to 40% sucrose, bottom loaded, and overlaid with two cushions of 35 and 8% sucrose. The gradient was centrifuged at 50,000 rpm in a TLS55 rotor (Beckman Coulter, Fullerton, CA) for 1 h. Light membranous organelles and membrane-associated proteins floated to the 35/8 interface, whereas heavier membranes and mitochondria are found in the pellet. Equal amounts of protein from each fraction were analyzed by SDS-PAGE and Western blotting. For flotation analysis of S2 cells, *arp1* dsRNA-treated cells and GFP dsRNA-treated cells were homogenized in homogenization buffer (HB; 8% sucrose and 3 mM imidazole, pH 7.4). A PNS was prepared and membrane fractionation was performed as described above. Fractions were collected, and equal amounts of proteins were analyzed by SDS-PAGE and Western blotting.

Real-Time Movies and Quantification

Amyloid precursor protein (APP) was C-terminally tagged with yellow fluorescent protein (YFP) and expressed under GAL4/UAS control using the P(Gal4)SG26.1 line (Kaether *et al.*, 2000; Gunawardena *et al.*, 2003). Crosses were made and female larvae with the genotype UAS-APP-YFP/+; P(Gal4)SG26.1/+; *arp1*¹/*arp1*¹ were filleted in a calcium free dissection buffer. Larvae were positioned with the ventral ganglion on the left and the segmental nerves projecting to the right. Movies were recorded using an Eclipse TE-2000-U inverted microscope (Nikon, Tokyo, Japan) at 100 $\times$ magnification at 10 frames per second and a CoolSNAP HQ cooled charge-coupled device camera driven by a MetaMorph imaging system version 5.0 (Molecular Devices, Sunnyvale, CA). We recorded 150 frames for each movie at 10 frames/s and 2×2 binning. For mitochondria, the leader peptide plus two amino acids (23 amino acids in toto) of human cytochrome oxidase subunit 8 was fused to GFP and expressed under GAL4/UAS control using P(GawB)D42 line. Because many mitochondria are stationary, a zone of bleached fluorescence was generated, and mitochondrial movement into the bleached zone was assessed using an MRC600 confocal microscope to collect 300 frames at a rate of 1 frame/s. For APP-YFP, kymographs were generated from movies using MetaMorph software and analyzed using MetaMorph and MATLAB 7.0 (MathWorks, Natick, MA). We determined particle populations, velocities, pause frequencies, and reversal frequencies based on the coordinates of each track traced on a particular kymograph. Net velocity was defined as the net distance traveled divided by the time elapsed during an entire movie (15 s). Segment velocity was defined as the total distance traveled by a particle during a particular run, divided by the duration of the run. A run could be

Figure 1. Axonal transport and the dynactin complex are disrupted in *arp1* mutant larvae. (A and B) In contrast to wild-type larvae *arp1* mutant animals exhibit the “tail-flip” phenotype common to axonal transport mutants in *Drosophila*. (C and D) Immunofluorescence with antibodies recognizing the synaptic cysteine string protein revealed abundant large accumulations within the segmental nerves of the *arp1* mutant, whereas wild-type animals exhibited typical background staining. (E) Larval brain extracts from wild-type larvae and homozygous *arp1* larvae were analyzed by Western blotting. The blots were probed with antibodies to different subunits of dynein and dynactin complexes. Tubulin antibody was used as a loading control. The p150^{Glued} and p50 subunits are obviously reduced in the *arp1* mutant larvae. (F) The dynactin complex is disrupted in the *arp1* mutant. Third instar brains were homogenized, and a high-speed supernatant was sedimented on a 5–20% sucrose gradients. In wild type, sedimentation of the dynactin subunits peaks at ~17S, whereas in the *arp1* mutant, both Arp1 and p50 sediment at ~4S–8S. Asterisk (*) points to dynactin in the Arp1 blot (the same blot has been reprobbed). The peak of Arp1 reactivity detected at ~4S in addition to the expected peak at 17S is due to cross reactivity of our Arp1 antibody with actin (see *Materials and Methods* and Figure 2).

terminated by a pause, reversal, or the end of the movie, and a run was a minimum of 10 frames (1 s) in length. Although run lengths were used to calculate velocities and determine pause or reversal points, we do not report explicit run lengths (cf. Gross *et al.*, 2002) because of significant inaccuracies resulting from runs already underway at the beginning of a movie, or observations that are prematurely terminated at the end of a movie. Instead, we assume that pauses reflect the dissociation of a motor from a microtubule after taking a number of discrete steps, and therefore, pause frequency per unit distance serves as an *indirect* measure of processivity. We defined pauses as an absence of directed movement ($<0.1 \mu\text{m}/\text{frame}$ in either direction) for at least 1 s for all moving particles. We report pause frequency as the number of pauses per micrometer of distance traveled by a given particle. Reversals were defined as a sustained change in direction of motion for at least 1 s; brief changes in direction occurring at subsampling scales were not detectable by the analyses. Stationary particles were defined as particles moving a distance of $<1.5 \mu\text{m}$ ($0.1 \mu\text{m}/\text{frame}$ in either direction) over the course of the entire 15-s movie. Thirty-three movies of axons from mutant larvae were analyzed, of which 16 movies had no particle motion whatsoever (i.e., only contained accumulations). Quantitative motion analysis was performed on the remaining 17 movies that did contain some moving particles. Some of these movies (11 of 17) revealed both moving particles and dense accumulations containing a large number of nonmoving particles. In these movies, because the number of stationary particles was impossible to count accurately, we set the maximum number of stationary particles per movie arbitrarily to 20. This procedure led to a conservative underestimate of the actual number of stationary cargoes. Only three wild-type movies were analyzed because the total number of moving particles in these three movies ($n = 107$) exceeded the number of moving particles in the 17 mutant movies ($n = 54$).

Online Supplemental Material

Online supplemental material describes detailed genetics experiments on *arp1* mutant and addresses whether dynactin is required for all dynein functions in *Drosophila*. Movies have been provided of APP-YFP particle motion in wild-type (FigS3videoA.mov) and *arp1* mutant (FigS3videoB.mov and FigS3videoC.mov) *Drosophila* segmental motor neuron axons. Kymographs in Figure 3, A–C, correspond to these videos. Additionally, movies of mitochondrial motion in wild-type (FigS3videoD.mov) and *arp1* mutant (FigS3videoE.mov) axons have been shown. Time-lapse panels from these movies are shown in Supplemental Figure 3, D and E. Imaging parameters are as described in *Materials and Methods*, with frames collected every 100 ms for 15 s for APP-YFP and every second for 300 s for mitochondria.

RESULTS AND DISCUSSION

Two *arp1* mutations were identified in a genetic screen for mutations that disrupt axonal transport in *Drosophila mela-*

nogaster larvae (Bowman *et al.*, 1999, 2000). The *arp1* mutants, like other known motor and motor-receptor mutants, exhibited a posterior larval paralysis phenotype (Figure 1, A and B) often accompanied by an upward tail flip during crawling (Hurd and Saxton, 1996; Bowman *et al.*, 1999, 2000; Martin *et al.*, 1999). We amplified and sequenced the *arp1* gene from genomic DNA of both mutant alleles. In one allele, *arp1*¹, we identified a point mutation in the *arp1* open reading frame that converted a conserved proline residue to a leucine at amino acid position 74 (Supplemental Figure 1). Although we were unable to identify a sequence change in the other mutant allele, *arp1*², it maps by recombination in the same region of chromosome 3 as *arp1*¹, and it fails to complement the *arp1*¹ mutant and deficiencies in this region of chromosome 3. Thus, we presume that this mutant affects a regulatory region whose sequence we have not delineated. Because both mutant alleles had similar phenotypes, we focused on the *arp1*¹ allele. To confirm that the *arp1*¹ larval phenotype is due to an axonal transport defect, we stained larval segmental nerves with antibodies to synaptic proteins such as cysteine string protein. In wild-type larvae, this antibody stains axons uniformly (Figure 1C), whereas in *arp1*¹ mutant larvae, synaptic proteins accumulate in axons (Figure 1D). The presence of these accumulations, previously observed in other axonal transport mutants (Bowman *et al.*, 2000), confirms that axonal transport is disrupted in the *arp1* mutants.

To ask whether dynactin is required for all dynein functions in *Drosophila*, we evaluated whether the consequences of loss of dynein and dynactin differ by comparing the phenotypes caused by loss of Arp1 to loss of DHC. Because both dynein and dynactin are thought to play essential roles at multiple stages in the cell cycle, we evaluated cell cycle defects in larval brains by using 5-bromo-2'-deoxyuridine (BrdU) incorporation, terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining, and by directly examining dividing neuroblasts stained with 4',6-diamidino-2-phenylindole. Observation and quantitation revealed that although the exact frequencies of defects in *arp1*

Table 1. Summary of phenotypic comparisons between *arp1* and *dhc* mutants

Process	Assays	Results	
		<i>arp1¹/arp1¹</i>	<i>dhc64C⁶⁻¹⁰/dhc64C⁶⁻¹⁰</i>
Cell cycle	Mitotic index and phenotype	Reduced mitotic index (decreased metaphase and prometaphase)	Altered mitotic distribution (increased metaphase, decreased prometaphase)
	BrdU incorporation	Abnormal mitotic figures Reduced incorporation	Abnormal mitotic figures Reduced incorporation
	Somatic cell viability in mitotic clones	Disorganization of cycling cells Complete lethality	Disorganization of cycling cells Complete lethality
Apoptosis	TUNEL	35× increase	6× increase
Oogenesis	Germline clones	16 nurse cells and no oocyte (oocyte does not differentiate)	16 nurse cells and no oocyte (oocyte does not differentiate)

Methods and data supporting this table are in Supplemental Material.

and *dhc* mutants were not identical, they were similar in character (Table 1 and Supplemental Figures 1 and 2). Similarly, failures in cell viability and oocyte determination evaluated in mitotic clone experiments were similar in *arp1* and *dhc* mutants (Table 1 and Supplemental Figures 1 and 2). These data suggest that dynein requires the dynactin complex for all cellular and developmental functions that we were able to test. We note that *arp1* mutant animals survive later in development than do *p150^{Glued}* mutants; this difference is likely due to different levels of maternal contribution of these two dynactin components.

To determine whether the *arp1¹* mutation destabilizes other dynactin subunits, we probed Western blots of larval brain homogenates with antibodies to different subunits of dynein and dynactin (Figure 1E). Although the amounts of DHC and dynein IC were unchanged in the *arp1¹* mutants compared with wild-type, the *p150^{Glued}* protein was virtually absent in the *arp1¹* mutant. The level of the dynamitin subunit (p50) was also significantly reduced. The observed reduction of the *p150^{Glued}* and p50 subunits suggests that the dynactin complex is disrupted in the *arp1¹* mutants. To test further whether *arp1¹* fully disrupts the dynactin complex, we performed sucrose density gradient analyses (Figures 1F and 2, C–F). Previous work has shown that p50, *p150^{Glued}*, and Arp1 all sediment at ~18S, indicating that they exist as a complex (Paschal *et al.*, 1993; Clark and Meyer, 1994; Eckley *et al.*, 1999). We observed that in wild-type larval brains, the dynactin complex subunits p50 and Arp1 sediment in a major peak at ~17S, as expected. In the *arp1¹* mutant, however, there is a significant shift such that the dynactin subunit p50 occurs in a peak at ~4S–8S, suggesting that the *arp1¹* mutants cause complete disruption of the dynactin complex. The *arp1¹* mutation leads to the disappearance of the Arp1 peak at 17S, whereas a peak at ~8S due to cross-reaction of our antibody with actin is still visible (Figures 1F and 2, C and D; see *Materials and Methods*).

To confirm and extend these observations, we used a *Drosophila* S2 cell culture system. S2 cells were grown in the presence of *arp1* dsRNA or a *GFP* dsRNA as a negative control. Soluble (HSS) and membrane (HSP) fractions were analyzed by Western blotting. As observed in brains of *arp1¹* mutant larvae, reduction of Arp1 mRNA (Figure 2B) and protein levels (Figure 2A) induces substantial reductions in the protein levels of both *p150^{Glued}* and p50, whereas the levels of DHC and KHC remain unchanged. We then performed sucrose density gradient analyses. To detect p50 and *p150^{Glued}* in the *arp1* dsRNA-treated samples, longer expo-

sure times were required. In both the soluble and membrane fractions, *arp1* dsRNA treatment caused p50 and *p150^{Glued}* subunits to shift from ~17S to ~8S, whereas DHC and KHC remained unchanged, peaking at 17S and 8S, respectively, in both conditions (Figure 2, C–F). In the control, *GFP* dsRNA sample, the soluble dynactin subunits did not completely overlap in one single peak, whereas the membrane associated dynactin subunits displayed partial cofractionation such that Arp1 primarily cofractionated with DHC, and *p150^{Glued}* primarily cofractionated with p50. Why the two sets of proteins have slightly different sedimentation profiles is unclear and might be caused by the stringency of the experimental conditions used or by a previously unsuspected instability of the soluble dynactin pool in S2 cells. Importantly, however, loss of Arp1 causes a clear disruption of the dynactin complex, such that the fractions that do contain DHC do not overlap with fractions containing dynactin subunits. Thus, similar to dynamitin overexpression (Echeverri *et al.*, 1996) (Eckley *et al.*, 1999), our results show that Arp1 mutation or deletion leads to disruption of the dynactin complex, in addition to reduction in the levels of both *p150^{Glued}* and p50. Our results also suggest that the membrane-associated dynactin complex is more stable than the soluble dynactin complex in S2 cells.

To test the hypothesis that dynactin is required to enhance the processivity of dynein, we investigated the effects of loss of Arp1 function on *in vivo* vesicle and organelle motility. We took advantage of the Gal4/UAS system to induce expression of a vesicular or a mitochondrial protein fused to YFP and GFP, respectively. For vesicles, we studied axonal transport of the vesicular APP fused to YFP (Kaether *et al.*, 2000; Gunawardena *et al.*, 2003) by using the P(Gal4)SG26.1 driver line, which is only expressed in a few motor neurons in the segmental nerves of third instars, allowing us to observe movements clearly. For mitochondria, we used a fusion of the leader peptide of human cytochrome c oxidase subunit 8 to GFP to target GFP to mitochondria (Horiuchi *et al.*, 2005; Pilling *et al.*, 2006) and used the P(GawB)D42 driver line, which is expressed in all motor neurons. If dynactin has a role in enhancing the processivity of dynein, we expected that in an *arp1* mutant background, APP-containing vesicles and mitochondria would move toward the cell body with shorter runs and more frequent pauses. In control animals, kymograph analysis revealed many movements of fluorescent APP particles toward and away from the cell body (Figure 3A), suggesting that these vesicles are powered by both anterograde and retrograde motors. In the *arp1¹* mu-

Figure 2. The dynactin complex, but not dynein, is disrupted in the *arp1* dsRNA-treated cells. (A) HSS and HSP were prepared from *arp1* dsRNA and GFP dsRNA-treated cells. Western blot analysis shows that loss of *arp1* leads to reduction in the levels of both p150^{Glued} and p50, whereas KHC and DHC remain unchanged. Note that in the HSS sample, tubulin and actin shown as loading controls display a lower level in the GFP control compared with the Arp1-treated sample, which explains the lower levels of DHC and KHC in that sample. Although the knockdown of Arp1 protein is evident in the HSP where no actin is detected, in the HSS, the Arp1 antibody cross-reacts with actin (see *Materials and Methods*). (B) Quantitation of transcript amounts was done with RT-qPCR. Arbitrary values of transcripts levels were obtained from duplicate data points and changes in transcript levels for the *arp1* RNAi-treated samples were compared with the GFP RNAi-treated samples and normalized to GAPDH. Knockdown of *arp1* transcript is shown as percentage (mean of 3 experiments $\pm$ SEM). (C–F) HSS and HSP from *arp1* dsRNA- and GFP dsRNA-treated cells were sedimented on a 5–20% sucrose gradients. In GFP dsRNA-treated cells, sedimentation of the dynactin subunits Arp1, p150^{Glued}, and p50 are found in a broad peak at ~17, whereas in the *arp1* dsRNA-treated cells, Arp1 levels are reduced and both p150^{Glued} and p50 are found at ~8S. Note that in the *arp1* dsRNA-treated samples, the levels of p50 and p150^{Glued} are reduced, and Western blots for p50 and p150^{Glued} required longer exposure times in D and F (see brackets and A). The Arp1 band detected at ~8S in both GFP and *arp1* dsRNA HSS corresponds to the actin cross-reactivity. This band is not observed in the HSP fraction where no actin can be detected. Dynein and kinesin sedimentation properties are not affected by reduction of Arp1, peaking at ~17S and ~8S, respectively. In C and D, arrowhead points to the 400-kDa dynein heavy chain.

tant, however, we observed significant numbers of stationary particles and organelle accumulations (Figure 3, B and C, and Supplemental Figure 3, B and C), as we had seen previously with immunofluorescence studies (Figure 1D). Surprisingly, we saw very little movement of APP particles in either direction in the axons, even in regions where no organelle accumulations were present (Figure 3D). In almost half of the axons studied (16 of 33 movies; see *Materials and Methods*), axons showed no directed movements of APP vesicles; moreover, we frequently observed that many particles, especially within accumulations, underwent apparent Brownian motion indicative of motor detachment from microtubules.

To quantify in vivo motor function in the remaining axons that did display limited particle motion (17 movies), we evaluated four parameters: net velocity, segment velocity, pause frequency, and reversal frequency. Net velocity was defined as the net distance traveled by a particle divided by the time elapsed during an entire movie. Segment velocity was defined as the distance traveled by a particle during a run (i.e., unbroken by a pause, reversal, or end of a movie) divided by the time elapsed during that run. Run lengths

were not explicitly reported due to inaccuracies resulting from runs already underway at the beginning of a movie, or prematurely terminated at the end of a movie. Rather, given the implicit correlation between shortened run lengths and increased likelihood of pausing, we used pause frequency, defined as the number of pauses occurring per micrometer of particle movement, as an indirect measure of processivity (see *Materials and Methods*). Reversals were indicated by a sustained change in direction of motion for at least 1 s.

The moving particles in the *arp1*¹ mutant animals had a much slower segment velocities than in wild type and traveled shorter distances (i.e., displayed slower net velocities) (Figure 3, E–G). Processivity was also affected in both directions, because there was an increased number of pauses per micrometer traveled in *arp1* axons compared with wild type (Figure 3H). Estimating run lengths revealed that only 5% of anterograde runs in the *arp1*¹ mutant animals were longer than 13 μ m, whereas fully 40% of anterograde runs in wild-type were longer than 13 μ m and 18% were 25 μ m or greater. Retrograde runs had similar behavior; only 3% of retrograde runs in the *arp1*¹ mutant animals were longer than 13 μ m, whereas fully 40% of retrograde runs in wild-

Figure 3. Vesicle movement is reduced in the *arp1*¹ mutant. (A) Kymograph from control movie displaying bidirectional movement in a dense field of APP-YFP particles. Bar, 10 μm . Movies and additional kymographs may be found in Supplemental Material and in Supplemental Figure 3. (B and C) Kymographs from *arp1*¹ larvae displaying altered patterns of movement. Kymograph in (B) shows a reduced number of APP-YFP particles, which are primarily stationary, with a few anterograde particles moving at velocities comparable to those in wild-type larvae (arrows). Kymograph in C shows primarily stationary particles, with some particles moving at a reduced velocity. Bar, 10 μm . Movies and additional kymographs may be found in Supplemental Material and in Supplemental Figure 3. (D) Populations of anterograde, retrograde, and stationary particles in *arp1*¹ mutant (gray) and wild-type (white) axons. (n = 284 particles analyzed from 17 *arp1*¹ mutant movies; n = 140 particles from 3 wild-type movies). (E) Net anterograde and retrograde velocities for moving particles in the *arp1*¹ mutant (gray) and wild-type (white) axons (mean $\pm$ SEM). (F) Cumulative frequency (defined as the summed percentage of particles with velocities less than or equal to velocity value on the x-axis) distribution of anterograde segment velocities of particles in *arp1*¹ mutant (circle) and wild-type (triangle) axons. (G) Cumulative frequency distribution of retrograde segment velocities of particles in *arp1*¹ mutant (circle) and wild-type (triangle) axons. (H) Pause frequency of *arp1*¹ mutant (gray) and wild-type (white) particles per micrometer of movement (mean $\pm$ SEM). Analysis excludes particles that do not pause. (I) Distribution of particle reversal frequency for *arp1*¹ mutant (gray) and wild-type (white) axons in anterograde and retrograde directions, expressed as frequency per total number of moving cargoes. Numbers above bars indicate sample size.

type were longer than 13 μm and 5% were 25 μm or greater. No retrograde runs in the *arp1*¹ mutant animals were longer than 19 μm , whereas 11% of retrograde runs in wild-type were longer than 19 μm . These quantitative analyses suggest that the slower net velocities in both anterograde and retrograde particle pools are a result of both slower segment velocities as well as increased pause frequency. (Figure 3, B, C, D, and H). Our analysis of a limited number of particle reversals indicates a trend toward increased reversal from anterograde to retrograde movement in *arp1*¹ mutant axons, suggesting an additional role of dynactin in coordinating the activity of opposing motors on the same particle (Figure 3I).

Anterograde and retrograde mitochondrial movement was also reduced in the *arp1*¹ mutant compared with wild type (Supplemental Figure 3, D and E). This is in contrast with the recent observation that a dominant negative allele of p150^{Glued} showed an increase in anterograde and retrograde movement of mitochondria (Pilling *et al.*, 2006). Thus, although our biochemical analyses demonstrate that *arp1*¹ mutations lead to disruption of the dynactin complex, heterozygous expression

of a dominant-negative allele of p150^{Glued} must retain sufficient function for mitochondrial transport (McGrail *et al.*, 1995).

A trivial cause of the lack of particle movement within the axons of *arp1*¹ mutant larvae could be that *arp1*¹ mutant neurons or animals are sick or dying. Two observations argue, however, that the motility defects observed are specifically caused by loss of dynactin function on vesicles or organelles. First, in previous work we found that intentionally inducing neuronal cell death did not cause defects in vesicle transport (Gunawardena *et al.*, 2003). Second, we observed that *arp1*¹ mutant siblings that were not dissected for live imaging survived at least another 48 h. Thus, although we cannot fully exclude the possibility of transport defects secondary to global neuronal toxicity, it seems likely that the loss of a functional dynactin complex has a direct effect on organelle processivity and motility.

To test directly whether dynactin is required to link dynein to membranous organelles, we examined the amount of membrane-bound dynein in *arp1*¹ mutant animals and in *arp1* dsRNA-treated S2 cells. We performed subcellular frac-

Figure 4. Dynein association with membranes is not affected by disruption of the dynactin complex. (A) Schematic representation of the flotation experiment. (B) Subcellular fractionation of third instar membranes on sucrose step gradients indicates that dynein is present in both soluble fraction and membrane fractions (35/8 interface) and that the amount of membrane-associated dynein is not altered in the *arp1¹* mutant larvae. Rab8 and syntaxin are used as loading controls and syntaxin is used as a membrane control. (C) Similar results were obtained when dynein association with membranes is analyzed in *arp1* dsRNA-treated S2 cells. Golgi membranes (d120) are enriched in the 35/8 interface, whereas mitochondria (cyt c) are enriched in the heavy membrane fraction. Importantly, as seen in A, levels of membrane-associated dynein are equivalent in both control and *arp1* RNAi-treated cells. Note that actin is present in all fractions and is thus masking the Arp1 reduction detected with the Arp1 antibody in the *arp1* dsRNA-treated cells (see Figure 2 and *Materials and Methods*).

tionation of larval brains or S2 cell extracts by membrane flotation on sucrose step gradients (Figure 4A). We observed no significant difference in the amount of dynein that was associated with the membranes enriched in the 35/8 sucrose interface in wild-type compared with the *arp1¹* mutant brains (Figure 4B). Membrane-associated proteins such as syntaxin were mainly present in the membrane fraction and not in the soluble pool, as expected. To confirm and extend these observations, we also analyzed membrane fractions prepared from *arp1* dsRNA-treated S2 cells. As reported in Figure 2A, and similar to what we saw with larval brains, we found that the levels of both p50 and p150^{Glued} are substantially reduced in membrane fractions in the 35/8 sucrose interface from the *arp1* dsRNA-treated S2 cells (Figure 4C). Although the overall levels of membrane associated DHC are lower in S2 cells than in larval brains, we consistently found that in both control and *arp1* dsRNA-treated cells, equivalent amounts of DHC are associated with membrane fractions enriched in Golgi and mitochondria. This result confirms the observation that in *arp1* dsRNA-treated S2 cells, the amount of dynein associated with a membrane fraction prepared by high-speed centrifugation is not altered (Figure 2A). Our results thus suggest that recruitment of dynein to membranous organelles, including Golgi and mitochondria

enriched fractions, is independent of an intact dynactin complex. It is possible, however, that smaller amounts of a disrupted dynactin complex on membranous compartment is sufficient to recruit DHC. In this regard, it is worth noting that the membrane-associated pool of p50 partially cofractionates with DHC in the absence of p150^{Glued} and Arp1 (Figure 2F). Although DHC might be recruited onto membranes by binding to a p50 subcomplex, this binding might not be efficient as suggested by our analyses of moving vesicles and organelles.

Our results suggest that an intact dynactin complex is not required for dynein to bind to membranous compartments. This proposal is supported by previous work suggesting that dynein subunits can interact directly with different types of cargo proteins (Almenar-Queralto and Goldstein, 2001). Our finding that *arp1* mutants exhibit numerous phenotypic defects that are similar to dynein mutants as well as obvious defects in minus-end-directed movements is consistent with the proposal that dynactin plays an important role in regulating the processivity, or other aspects, of dynein motility (King and Schroer, 2000). However, even though we see apparently unchanged amounts of dynein binding to membranes when dynactin is disrupted, it is formally possible that the mode of attachment of dynein to

cargo influences the characteristics of motility. For example, altered dynactin complex behavior resulting from Arp1 reduction on moving vesicles and organelles might cause incorrect attachment of DHC to membranous organelles, without affecting processivity per se. Similarly, the increased number of stationary organelles observed after Arp1 reduction could be explained by the direct association of dynein with membranes in a nonphysiological manner. The observed decrease in retrograde velocity may also reflect abnormal attachment of the dynein complex to membranes. Alternatively, given that dynein is a cooperative motor, this decrease in velocity may also be a result of poor coordination among dynein complexes attached to a given cargo. We are unable to distinguish between these scenarios based on our data thus far.

Our observation that the *arp1¹* mutant also disrupts anterograde movement of APP-containing vesicles and mitochondria does not easily fit the proposal that the regulatory function of dynactin is restricted to the processivity of dynein. Interestingly, depletion of Arp1 may have reduced the levels of KHC on membranes (Figure 4C). Although the degree of reduction is small, it may suggest how dynactin functions in anterograde movement. Whether dynactin in fact plays a direct role in kinesin recruitment to membranes will require further investigation. Nonetheless, our data are consistent with an additional role for dynactin in regulating anterograde motors such as kinesin, or as recently proposed, a role in coordinating plus and minus-end-directed transport (Gross *et al.*, 2000, 2002a,b). In particular, a dominant mutation in p150^{Glued} severely impaired “anterograde” motion of lipid droplets in *Drosophila* embryos. Similarly, disruption of dynactin by overexpressing dynamitin inhibited movement of endosomes in both directions (Valetti *et al.*, 1999). Finally, biochemical support for a role of dynactin in coordinating plus- and minus-end-directed movements comes from the finding that a subunit of kinesin II can interact with p150^{Glued} (Deacon *et al.*, 2003) and that dynein interacts directly with kinesin light chain (Ligon *et al.*, 2004). Thus, our data, combined with earlier work, suggests that the *in vivo* function of dynactin is to regulate and/or coordinate bidirectional motility, but that dynactin may not be required to link dynein to membranes.

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