Mycalolide B dissociates dynactin and abolishes retrograde axonal transport of dense-core vesicles

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ABSTRACT Axonal transport is critical for maintaining synaptic transmission. Of interest, anterograde and retrograde axonal transport appear to be interdependent, as perturbing one directional motor often impairs movement in the opposite direction. Here live imaging of *Drosophila* and hippocampal neuron dense-core vesicles (DCVs) containing a neuropeptide or brain-derived neurotrophic factor shows that the F-actin depolymerizing macrolide toxin mycalolide B (MB) rapidly and selectively abolishes retrograde, but not anterograde, transport in the axon and the nerve terminal. Latrunculin A does not mimic MB, demonstrating that F-actin depolymerization is not responsible for unidirectional transport inhibition. Given that dynactin initiates retrograde transport and that amino acid sequences implicated in macrolide toxin binding are found in the dynactin component actin-related protein 1, we examined dynactin integrity. Remarkably, cell extract and purified protein experiments show that MB induces disassembly of the dynactin complex. Thus imaging selective retrograde transport inhibition led to the discovery of a small-molecule dynactin disruptor. The rapid unidirectional inhibition by MB suggests that dynactin is absolutely required for retrograde DCV transport but does not directly facilitate ongoing anterograde DCV transport in the axon or nerve terminal. More generally, MB’s effects bolster the conclusion that anterograde and retrograde axonal transport are not necessarily interdependent.

INTRODUCTION

The formation and maintenance of nerve terminals depend on anterograde axonal transport from the soma. Although distinct microtubule-based motors are used for anterograde and retrograde transport, numerous studies have shown that anterograde and retrograde axonal transport exhibit interdependence. Specifically, genetic and antibody-based perturbations have shown that inhibiting kinesin, dynein, or dynactin, a large dynein accessory complex required for initiation of retrograde transport in the nerve terminal (Schroer, 2004; Lloyd et al., 2012; Moughamian and Holzbaur, 2012), impedes organelle transport in both directions (Waterman-Storer et al., 1997; Martin et al., 1999; Deacon et al., 2003; Pilling et al., 2006; Haghnia et al., 2007; Kwinter et al., 2009; Park et al., 2009). This bidirectional interdependence is not understood at the molecular level but might have great relevance to dynactin’s contribution to neurological diseases (Puls et al., 2003; Ström et al., 2008; Farrer et al., 2009).

An important organelle for synaptic function and a classic model of an anterograde cargo is the dense-core vesicle (DCV), which in neurons contains neuropeptides, secreted enzymes, and neurotrophins. Neuronal DCV function depends on its anterograde transport in axons and the nerve terminal to sites of release (e.g., synaptic boutons). However, DCVs do not move in one direction (Wong et al., 2012). Their bidirectional transport likely facilitates turnover of the presynaptic pool of DCVs and ensures uniform delivery to multiple release sites by long-distance vesicle circulation.
kelley linked (waterman-storer) grade transport of axonal organelles, including DCVs, is functionally previously reports that anterograde and dynactin-dependent retrograde inhibition by MB (Figure 2, A–C). However, in contrast to neurons, retrograde flux of DCVs containing BDNF-mRFP was dramatically inhibited by MB (Figure 2, A–C). This is not due to an effect on F-actin but instead reflects the ability of MB to dissociate the dynactin complex.

Thus a novel, small-molecule dynactin inhibitor is identified to provide new evidence that anterograde and retrograde axonal transport of DCVs are not necessarily interdependent.

**RESULTS**

Delivery of neuromodulatory neuropeptides to the nerve terminal is based on vesicle circulation, which includes switching from retrograde to anterograde axonal transport in the proximal axon near the soma (Wong et al., 2012). To determine whether F-actin, which is abundant at the axon initial segment, is involved in this directional change, the F-actin–depolymerizing sponge macrolide toxin MB (Saito et al., 1994) was applied to filleted Drosophila larva expressing green fluorescent protein (GFP)-tagged atrial natriuretic factor (ANF-GFP), a cargo for DCVs (Burke et al., 1997; Han et al., 1999; Rao et al., 2001). To examine retrograde motility of DCVs, the soma and proximal axon of the lateral tracheal dendrite sensory neuron was photobleached to eliminate the signal from anterograde transport of DCVs in this region. Then retrograde transport of DCVs was monitored selectively by measuring the number of DCV puncta that appeared in the soma and proximal axon (Wong et al., 2012). Remarkably, treatment with 2 μM MB for 20 min abolished this fluorescence recovery after photobleaching (FRAP; Figure 1, A and B), indicating that retrograde axonal transport of DCVs was inhibited.

To determine whether the MB effect was specific to the movement of ANF-GFP puncta in the proximal axon of Drosophila neurons or was due to the FRAP procedure we used, we measured transport of another DCV cargo, monomeric red fluorescent protein (mRFP)-tagged brain-derived neurotrophic factor (BDNF), in cultured rat hippocampal neuron axons with live-cell imaging (Figure 2A). Consistent with the FRAP results using ANF-GFP in Drosophila neurons, retrograde flux of DCVs containing BDNF-mRFP was dramatically inhibited by MB (Figure 2, A–C). However, in contrast to previous reports that anterograde and dynactin-dependent retrograde transport of axonal organelles, including DCVs, is functionally linked (Waterman-Storer et al., 1997; Martin et al., 1999; Pilling et al., 2006; Haghnia et al., 2007; Kwinter et al., 2009; Park et al., 2009), MB inhibition of retrograde transport occurred without inhibition of overall axonal transport flux (Figure 2, B and C and Supplemental Movie S1). Further, DCV velocity and run length were reduced only for retrograde axonal transport (Table 1). Therefore MB inhibits retrograde DCV transport while preserving anterograde transport in mammalian axons.

MB acts in vitro to depolymerize purified F-actin (Saito et al., 1994). Furthermore, phalloidin labeling established that MB depolymerizes F-actin in nerve growth factor (NGF)-differentiated rat PC12 cells (Ng et al., 2002a,b), which feature polarized microtubules and bidirectional DCV transport in their neurites (Lochner et al., 1998; Kelley et al., 2010). Therefore, to determine whether retrograde DCV transport inhibition by MB was due to loss of F-actin, we treated differentiated PC12 cells and hippocampal neurons with another actin poison, latrunculin A (LatA). Phalloidin labeling confirmed the loss of F-actin structures and the preservation of DCVs in both LatA-treated PC12 cells and hippocampal neurons (Figure 3, A and B). However, in contrast to the effect of MB in neurons, retrograde DCV flux in PC12 cells was not inhibited by LatA (Figure 3C). Anterograde and retrograde axonal transport of DCVs were also comparable in hippocampal neurons treated with LatA (Figure 3D and Table 1). Hence, F-actin depolymerization does not account for...
MB’s selective inhibition of retrograde DCV transport in axons and neurites, suggesting that MB might have an alternative target. MB is chemically related to a number of F-actin–severing macroside toxins. The amino acids that contact these toxins in rabbit skeletal muscle actin have been identified by x-ray crystallography (Klenchin et al., 2003; Hirata et al., 2006). Strikingly, many of these are conserved in actin-related protein 1 (Arp1), the most closely related protein to actin; for example, >80% of toxin-interacting amino acids in rabbit muscle actin are identical in rat Arp1, with the rest being conserved changes (F for Y; T for S). Arp1, which is the only actin-related protein that can form filaments, is found only as a component of dynactin, a large, multiprotein dynein accessory complex (Schroer, 2004) that facilitates efficient initiation of retrograde axonal transport of DCVs and other organelles at the distal nerve terminal ending (i.e., the most distal synaptic bouton; Lloyd et al., 2012; Moughamian and Holzbaur, 2012). Therefore we investigated whether MB affects dynactin function by measuring retrograde transport from the most distal synaptic bouton within a branch of the Drosophila neuromuscular junction.

Specifically, we measured the effect of MB on DCV transport in type Ib motor neuron terminals of the larval Drosophila neuromuscular junction. For anterograde transport, a series of distal boutons was photobleached, and FRAP was used to monitor anterograde transport into the bleached region. Specifically, flux was measured as the number of DCVs entering the photobleached region per minute. As can be seen based on the reappearance of fluorescent puncta in the photobleached region (Figure 4A) and quantification of anterograde flux (Figure 4C, black bar, Antero), MB did not affect anterograde DCV transport in the nerve terminal. To assay retrograde transport out of the most distal bouton, we used simultaneous photobleaching.

<table>
<thead>
<tr>
<th>Traffic values</th>
<th>Anterograde</th>
<th>Retrograde</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flux (min⁻¹)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>4.54 ± 0.58</td>
<td>3.35 ± 0.34</td>
</tr>
<tr>
<td>Mycalolide B</td>
<td>5.72 ± 0.82</td>
<td>0.07 ± 0.03***</td>
</tr>
<tr>
<td>Latrunculin A</td>
<td>4.88 ± 0.56</td>
<td>5.17 ± 0.62**</td>
</tr>
<tr>
<td>Velocity (μm/s)</td>
<td>1.37 ± 0.06</td>
<td>1.42 ± 0.06</td>
</tr>
<tr>
<td>Mycalolide B</td>
<td>1.32 ± 0.09</td>
<td>0.35 ± 0.13***</td>
</tr>
<tr>
<td>Latrunculin A</td>
<td>1.26 ± 0.03</td>
<td>1.36 ± 0.05</td>
</tr>
<tr>
<td>Run length (μm)</td>
<td>6.41 ± 0.46</td>
<td>5.77 ± 0.28</td>
</tr>
<tr>
<td>Mycalolide B</td>
<td>10.19 ± 0.88***</td>
<td>1.81 ± 0.81***</td>
</tr>
<tr>
<td>Latrunculin A</td>
<td>5.56 ± 0.34</td>
<td>5.74 ± 0.35</td>
</tr>
</tbody>
</table>

Control (n = 40 kymographs, 40 cells, 2414 DCVs); mycalolide B (n = 22 kymographs, 22 cells, 633 DCVs); latrunculin A (n = 23 kymographs, 23 cells, 2089 DCVs).

**p < 0.01, when compared with control (from each column).
***p < 0.001, when compared with control (from each column).

TABLE 1: DCV axonal transport parameters in hippocampal neurons.

FIGURE 2: MB inhibits retrograde but not anterograde axonal transport of DCVs in cultured hippocampal neurons. (A) Left, BDNF-mRFP in hippocampal neuron soma and axon. Scale bar, 15 μm. Right, magnification of the boxed region to reveal axonal puncta. (B) Kymographs showing the MB effect on axonal transport. Green lines indicate anterograde transport, and red lines indicate retrograde transport. (C) Quantification of DCV flux in controls (Con, n = 40 cells) and MB (n = 22 cells). ***p < 0.001.
port of DCVs to the most distal bouton (i.e., by continuous photo-bleaching anterogradely transported DCVs). This allowed selective measurement of retrograde DCV transport out of the most distal bouton (i.e., the flux into the photobleached region from the most distal bouton). Under control conditions, retrograde DCV transport and imaging (SPAIM; Wong et al., 2012). For these experiments, the photobleached region was restricted to a series of proximal boutons, but the most distal bouton in the terminal branch was spared. Then a photobleaching beam was positioned adjacent to the most proximal photobleached bouton to prevent FRAP by anterograde transport of DCVs to the most distal bouton (i.e., by continuous photo-bleaching anterogradely transported DCVs). This allowed selective measurement of retrograde DCV transport out of the most distal bouton (i.e., the flux into the photobleached region from the most distal bouton). Under control conditions, retrograde DCV transport

**FIGURE 3:** LatA does not mimic the MB effect on retrograde transport. Differentiated PC12 cells (A) and hippocampal neurons (B) expressing fluorescent protein–tagged cargoes (ANF and BDNF, respectively) were treated with vehicle (Con) or 10 μM LatA for 20 min and fixed, and then F-actin was stained with Alexa 568 (A) or 488 (B) phalloidin (Ph-Alexa). Bars, 10 μm (A), 25 μm (B). (C) Comparison of the effects of MB and LatA on anterograde and retrograde DCV flux in PC12 cell neurites. Con (open bars, n = 13), MB (black bars, n = 6), LatA (blue bars, n = 9). ****p < 0.0001. (D) Anterograde and retrograde DCV flux in LatA-treated hippocampal axons (n = 23). Note that retrograde flux is comparable to anterograde transport in LatA, in contrast to the dramatic decrease in retrograde transport produced by MB in hippocampal neurons (Figure 2).
Because these results were obtained at the site where retrograde transport is initiated via dynactin (Lloyd et al., 2012; Moughamian and Holzbaur, 2012), they are consistent with the hypothesis that MB inhibits dynactin activity. Because MB depolymerizes F-actin, we hypothesized that it might also depolymerize the Arp1 filament to inhibit dynactin. Therefore we determined biochemically the effect of MB on the integrity of the dynactin complex, which also includes a single actin protomer, Arp1, CapZ α and β, p62, p25, p27, p150\textsuperscript{Glued}, p24, and dynamitin (DM) subunits (reviewed in Schroer, 2004). First, we treated PC12 cell extracts with MB and monitored dynactin integrity using immunoprecipitation of p150\textsuperscript{Glued} followed by immunoblotting for (Supplemental Figure S1). Because these results were obtained at the site where retrograde transport is initiated via dynactin (Lloyd et al., 2012; Moughamian and Holzbaur, 2012), they are consistent with the hypothesis that MB inhibits dynactin activity.

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mented very slowly (Figure 6B), suggesting dissociation of the filament to monomers or dimers. The behavior of dynein IC, by contrast, was unaffected by MB. This indicates that MB disrupts the dynactin complex while sparing the integrity of the dynein motor.

To verify that MB can act directly on dynactin, we treated purified bovine dynactin with MB before sucrose gradient sedimentation and evaluated the behavior of dynactin components in the gradient fractions via Coomassie blue staining after SDS–PAGE. This method allowed detection of additional dynactin subunits, specifically CapZ, p25, and p24 (Figure 6C). In this experiment, Arp1 sedimented in a single peak at \( \sim 6S \), consistent with complete depolymerization of the filament (Figure 6D). Other components also exhibited S values consistent with dynactin disassembly (Figure 6D). We attribute the Arp1. Strikingly, MB profoundly diminished the amount of Arp1 co-immunoprecipitated with p150Glued (Figure 5A, Extracts; \( n = 5 \)). An effect was seen even at submicromolar concentrations (Figure 5B). MB had a similar effect when intact PC12 cells were treated with MB before washing, lyase preparation, and immunoprecipitation (Figure 5A, Cells, \( n = 3 \)). Given that this last protocol entailed removal of MB and then dilution during generation of the cell extract and immunoprecipitation, these results suggest that the dissociation of Arp1 from p150Glued induced by MB is long-lasting and possibly irreversible.

Next we used sucrose gradient sedimentation to examine the effects of MB on the stability of dynactin. To assay cellular dynactin, we treated HEK293T cell lysates with MB or vehicle (dimethyl sulfoxide [DMSO]) and fractionated using sucrose density gradient centrifugation. Dynactin subunits in the gradient fractions were then detected by immunoblotting. Dynactin IC, which normally cosediments at \( \sim 20S \) with dynactin, was assayed as a control. In samples treated with MB, components of dynactin’s shoulder–arm complex (e.g., p150Glued and dynamitin) and the pointed-end complex component p27 sedimented much more slowly than in the control, indicating that they were no longer associated with each other in the dynactin complex (Figure 6, A and B). This analysis also revealed evidence of Arp1 filament disassembly by MB, as approximately half of the Arp1 sedi-

FIGURE 5: MB dissociates the dynactin complex. (A) MB blocks co-IP of Arp1 with p150Glued. Vehicle or 2 μM MB was applied to PC12 cells before solubilization (Cells) or to PC12 cell extracts (Extracts). p150Glued immunoprecipitates were probed for p150Glued and Arp1. (B) Quantification of the concentration dependence of MB on dynactin integrity in cell extracts, assessed by co-IP with p150Glued. *p < 0.05, **p < 0.01, ***p < 0.001.

FIGURE 6: Analysis of MB on dynactin integrity by sucrose density centrifugation. (A, B) HEK293T cell lysate treated with vehicle (A) or 2 μM MB (B) was subjected to sedimentation into 5–20% sucrose gradients, and gradient fractions were analyzed by immunoblotting for dynactin subunits and the dynein IC. Densitometry indicates about half of the Arp1 in a monomer/dimer pool in the presence of MB vs. <5% in the vehicle control. (C, D) Analysis of MB on dynactin integrity in vitro. Purified bovine dynactin treated with vehicle (C) or 2 μM MB (D) was subjected to sedimentation into a 5–20% sucrose gradient, and gradient fractions were analyzed by PAGE (stained with Coomassie blue).
more dramatic MB effect with purified protein than in the cell lysate to the absence of cellular actin, which would compete for MB binding. Of most importance, this analysis demonstrates that MB interacts directly with and disrupts the dynactin complex, including its Arp1 filament.

**DISCUSSION**

The biochemical and live-neuron imaging studies presented here lead to several new insights into dynactin and axonal transport. First, MB is a novel dynactin inhibitor that potently and directly disrupts the dynactin complex. The other condition reported to have this effect is high concentration of the chaotropic salt potassium iodide (Eckley et al., 1999). The recently published dynactin structure (Urnauvicius et al., 2015) provides additional insights into how MB may trigger subunit release. The predicted MB-binding site on Arp1, based on interactions of similar macrolide toxins with actin (Klenchin et al., 2003; Hirata et al., 2006), is immediately adjacent to the site where the dynamin N-terminus binds Arp1. MB binding may thus dislodge dynamitin and the associated p150

netic experiments, and without introducing the steric hindrance of dynactin and a dynactin antibody, respectively (Waterman-Storer et al., 1997; Martin et al., 1999; Pilling et al., 2006; Hagnhia et al., 2007; Kwinter et al., 2009; Park et al., 2009). However, MB acts rapidly, which obviates the slow, indirect effects that can occur in genetic experiments, and without introducing the steric hindrance of antibody binding. A more subtle effect of dynactin on anterograde transport is suggested by the finding that MB, but not LatA, increased anterograde run length (Table 1). This further demonstrates the effectiveness of MB and suggests that dynactin has an attenuating effect on anterograde transport, which is very different from previous models. However, as no statistically significant effect was seen on anterograde flux (Table 1), the physiological importance of this observation is unclear, especially when compared with the accompanying dramatic inhibition of retrograde transport. Overall MB experiments suggest that dynactin’s function in axonal transport of DCVs should be redefined from bidirectional facilitator to being strictly required for retrograde transport without a direct requirement in anterograde transport.

The independent nature of anterograde and retrograde axonal transport revealed by MB treatment is reminiscent of what is seen when the dynein regulators Nudel and LIS1 are inhibited. In these cases, retrograde axonal transport of other (but not all) organelles is preferentially inhibited (Zhang et al., 2009; Yi et al., 2011). Therefore there are now multiple examples in which perturbation of dynein accessory components (dynactin, LIS1, or Nudel) does not also block plus end-directed axonal transport. Our work is unique in that MB is the first rapidly acting small-molecule inhibitor of retrograde transport to yield clear evidence that retrograde and anterograde transport in the axon and nerve terminal are not mechanistically linked.

**MATERIALS AND METHODS**

**Experimental preparations for imaging**

Rat hippocampal neuronal cell culture, transfections, imaging, and image analysis were performed as described previously, with flux being derived from kymographs and normalized for length of axonal region of interest (Kwint et al., 2009). The astrocyte feeder layer for the neuronal coculture was generated using neural progenitor cells as described previously (Miranda et al., 2012).

Drosophila lateral tracheal dendrite neurons (Bodmer and Jan, 1987) and muscle 6/7 type Ib motor neuron boutons were studied in UAS-ANF-GFP,386Y-GAL4 wandering third-instar larvae. To image actin with Lifeact (Riedl et al., 2008), females were crossed with male UAS-Lifeact-Ruby flies (Hatan et al., 2011). UAS-ANF-GFP (originally called UAS-preproANF-EMD) (Rao et al., 2001) and UAS-Lifeact-Ruby are available from the Bloomington Drosophila Stock Center (Bloomington, IN; stocks 7001 and 35544). Imaging, FRAP, and SPAIM were performed as described previously (Levitan et al., 2007; Wong et al., 2012) in Ca

Electrical stimulation was performed using a conventionalglass pipette connected to a stimulus generator (World Precision Instruments, Sarasota, FL; model S135C) and a monopolar microelectrode. Fluorescence was imaged using a Leica TCS SP5 microscope equipped with 488-nm-diode-pumped Argon and 568-nm-Laser diode-excited and 594-nm-Omega Longpass filter, respectively. Images were acquired using a 63× oil objective at 0.5-s intervals for 10 s before stimulation. After stimulation, images were acquired at 0.5-s intervals for 10 s. Images were analyzed using ImageJ software (available at https://imagej.nih.gov/ij/) and expressed as the change in fluorescence intensity compared to baseline (using the Leica SP5 software for ratio changes). Values are presented as means ± SEM.

**Chemicals**

Alexa Fluor–phalloidin (568 for PC12 and 488 for hippocampal neurons; Invitrogen, Carlsbad, CA) was used as directed by the manufacturer. DMSO 0.1% was used as the vehicle. We applied 2 μM mycocalicin B (Wako, Richmond, VA) or 10 μM latrunculin A (Sigma-Aldrich, St. Louis, MO) for 20 min at room temperature for Drosophila and at 37°C for hippocampal neurons.

**Immunoprecipitation**

PC12 cells were grown for 2 d with 10% fetal bovine serum in DMEM before treatment with MB or vehicle. For treatment of intact cells, the medium was removed, cells were washed with normal saline, and MB or DMSO was added in normal saline for 20 min at room
temperature. In all immunoprecipitation (IP) experiments, cells were solubilized in 100 mM HEPES, pH 7.4, 1 mM EGTA, 2 mM MgCl$_2$, 25 mM NaCl, 0.5 mM dithiothreitol, 1% Triton X-100, 1 mM fresh phenylmethylsulfonyl fluoride (PMSF), and Complete Mini EDTA protease inhibitor cocktail (Roche, Indianapolis, IN). Cells were then gently scraped off from the dish on ice, debris was pelleted by centrifugation at 10,000 × g at 4°C for 15 min, and the supernatant was then used for IP. For extract treatments, supernatants were warmed to room temperature before adding MB or DMSO for 20 min. For immunoblot analysis, protein concentrations were measured by a bicinchoninic acid protein assay kit (Thermo Scientific, Waltham, MA) with bovine serum albumin (BSA) as a standard. Samples containing equivalent amounts of protein (250–350 μg) were precleared with 30 μl of protein A/G plus agarose (Calbiochem, San Diego, CA) overnight at 4°C. Antibody–protein complexes were precipitated with 65 μl of protein A/G plus-agarose for 3 h at 4°C. Cold 1x phosphate-buffered saline (PBS) was used to wash agarose beads five times. Protein was then eluted with 50 μl of 2x reducing electrophoresis sample buffer and used for gel electrophoresis after denaturation at 95°C for 5 min. Gels were transferred onto a Trans-Blot Transfer Medium Pure Nitrocellulose Membrane (0.2 μm; Bio-Rad, Hercules, CA), followed by electroblotting. After blocking with 1% (wt/vol) BSA in PBST (0.1% Tween 20 in PBS) for 1 h at room temperature, the membrane was incubated 1 h at room temperature with 1 μg of anti-rabbit polyclonal against α-centractin (Arp1) antibody and 10 μl of mouse anti-p150$^{\text{Glued}}$ (BD Bioscience, San Jose, CA) overnight at 4°C. Antibody–protein complexes were precipitated with 65 μl of protein A/G plus-agarose for 3 h at 4°C. Cold 1x phosphate-buffered saline (PBS) was used to wash agarose beads five times. Protein was then eluted with 50 μl of 2x reducing electrophoresis sample buffer and used for gel electrophoresis after denaturation at 95°C for 5 min. Gels were transferred onto a Trans-Blot Transfer Medium Pure Nitrocellulose Membrane (0.2 μm; Bio-Rad, Hercules, CA), followed by electroblotting. After blocking with 1% (wt/vol) BSA in PBST (0.1% Tween 20 in PBS) for 1 h at room temperature, the membrane was incubated 1 h at room temperature with 1 μg of anti-rabbit polyclonal against α-centractin (Arp1) antibody and 10 μl of mouse anti-p150$^{\text{Glued}}$ (250 μg/ml). Membranes were washed four times for 5 min with PBST and then incubated for 1 h at room temperature with IRDye 800CW Gt anti-rabbit immunoglobulin G (iG; H + L) antibody and IRDye 680LT anti-mouse IgG (H + L) antibody (1:15,000; Li-COR Bioscience, Lincoln, NE). Infrared fluorescence was detected with the Odyssey Imaging System.

Sucrose density centrifugation
We incubated 50 μg of purified bovine dynactin (Bingham et al., 1998) or 250–500 μg of a HEK293T cell detergent lysate (20 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 1 mM EGTA, 2 mM β-ME, with protease inhibitors) for 30 min at room temperature with either vehicle (DMSO, 2% of final volume) or 2 μM MB (in DMSO) in a final volume of 300–500 μl in 35 mM Tris-Cl, 5 mM MgSO$_4$, pH 7.2 (TM buffer). The samples were then layered onto 11.8 ml of 5–20% sucrose (wt/vol) gradients in TM buffer and centrifuged for 17.5 h at 34,000 rpm in an SW41 rotor. Twelve 1-ml fractions were collected from each gradient. For the purified dynactin samples, the entirety of each fraction was subjected to TCA precipitation and then run on a 12.5% SDS polyacrylamide gel and transferred to PVDF membrane for immunoblotting. The following antibodies were used to detect dynactin subunits: mouse monoclonal antibodies 150B and 45A (Quinntyne et al., 1999; Schaf er et al., 1994), mouse anti-dynamitin (BD Biosciences), and rabbit anti-DCTN6 (to p27; Proteintech Group, Chicago, IL). The mouse monoclonal antibody 74.1 (EMD Millipore, Billerica, MA) was used to detect dynein IC as a sedentation standard and internal control.

Statistics
Error bars represent SEM. Statistical comparison for two experimental groups was based on Student’s t test. One-way analysis of variance with Dunnett’s post test was used for comparison to control for more than two experimental groups.

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