Regulation of microtubule-based transport by MAP4

Irina Semenova, Kazuho Ikeda, Karim Resaul, Pavel Kraikivski, Mike Aguiar, Steven Gygi, Ilya Zaliapin, Ann Cowan, and Vladimir Rodionov

R.D. Berlin Center for Cell Analysis and Modeling and Department of Cell Biology, University of Connecticut Health Center, Farmington, CT 06030; Quantitative Biology Center, RIKEN, Osaka 565-0874, Japan; Department of Biological Sciences, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061; Department of Cell Biology, Harvard Medical School, Boston, MA 02115; Department of Mathematics and Statistics, University of Nevada–Reno, Reno, NV 89557

ABSTRACT Microtubule (MT)-based transport of organelles driven by the opposing MT motors kinesins and dynein is tightly regulated in cells, but the underlying molecular mechanisms remain largely unknown. Here we tested the regulation of MT transport by the ubiquitous protein MAP4 using Xenopus melanophores as an experimental system. In these cells, pigment granules (melanosomes) move along MTs to the cell center (aggregation) or to the periphery (dispersion) by means of cytoplasmic dynein and kinesin-2, respectively. We found that aggregation signals induced phosphorylation of threonine residues in the MT-binding domain of the Xenopus MAP4 (XMAP4), thus decreasing binding of this protein to MTs. Overexpression of XMAP4 inhibited pigment aggregation by shortening dynein-dependent MT runs of melanosomes, whereas removal of XMAP4 from MTs reduced the length of kinesin-2–dependent runs and suppressed pigment dispersion. We hypothesize that binding of XMAP4 to MTs negatively regulates dynein-dependent movement of melanosomes and positively regulates kinesin-2–based movement. Phosphorylation during pigment aggregation reduces binding of XMAP4 to MTs, thus increasing dynein-dependent and decreasing kinesin-2–dependent motility of melanosomes, which stimulates their accumulation in the cell center, whereas dephosphorylation of XMAP4 during dispersion has an opposite effect.

INTRODUCTION Intracellular transport is essential for the delivery of membrane-bound organelles, RNA granules, and chromosomes to specific cellular destinations and is critical for diverse biological processes such as mitosis, membrane trafficking, cell locomotion, and spatial organization of the cytoplasm (Lane and Allan, 1998; Caviston and Holzbaur, 2006; Akhmanova and Hammer, 2010; Walczak et al., 2010; Kapitein and Hoogenraad, 2011; Stehbens and Wittmann, 2012; Foley and Kapoor, 2013). Tracks for long-range intracellular transport are provided by cytoplasmic microtubules (MTs) organized into a polarized radial array with minus ends clustered in the cell center and plus ends pointing to the cell surface. The inherent polarity of MTs is recognized by MT motors bound to the surface of cargo organelles and cytoplasmic particles. These motors—kinesins and dynein—use the energy of ATP hydrolysis to move cargoes specifically to the plus or minus ends of MTs (Vale, 2003). Kinesins generally support transport to the MT plus ends (Goldstein, 2001; Hirokawa et al., 2009; Verhey et al., 2011), whereas dynein is exclusively minus-end directed (Hook and Vallee, 2006).

Opposing MT motors are usually bound to the surface of the same cargo organelles, which results in the discontinuous (“saltatory”) pattern of their movement, with stochastic transitions among three states: uninterrupted displacements to the minus or plus ends, and pauses (Gross, 2004; Welte, 2004; Jolly and Gelfand, 2011). The balance between the plus and minus end–directed runs, which determines the direction of MT transport, is tightly regulated.

This article was published online ahead of print in MBoC in Press (http://www.molbiocell.org/cgi/doi/10.1091/mbc.E14-01-0022) on August 20, 2014. Address correspondence to: Vladimir Rodionov (rodionov@nso.uchc.edu).

Abbreviations used: MAP, microtubule-associated protein; MBD, microtubule-binding domain; MT, microtubule; XMAP, Xenopus microtubule-associated protein.

© 2014 Semenova et al. This article is distributed by The American Society for Cell Biology under license from the author(s). Two months after publication it is available to the public under an Attribution–Noncommercial–Share Alike 3.0 Unported Creative Commons License (http://creativecommons.org/licenses/by-nc-sa/3.0).

“ASCB®,” “The American Society for Cell Biology®,” and “Molecular Biology of the Cell®” are registered trademarks of The American Society of Cell Biology.
in cells. This regulation can involve changes in the numbers or the activation state of kinesin and dynein motors associated with cargoes (Kamal and Goldstein, 2002; Karcher et al., 2002; Verhey and Hammond, 2009; Akhmanova and Hammer, 2010; Barlan et al., 2013b) or modification of MT transport tracks (Verhey and Hammond, 2009). The state of MT tracks is influenced by specific posttranslational modifications of tubulin subunits (Liao and Gundersen, 1998; Reed et al., 2006; Dunn et al., 2008; Cai et al., 2009; Konishi and Setou, 2009) or nonmotor microtubule-associated proteins (MAPs).

MAPs are a group of proteins that bind along the length of MTs, promote their assembly, and stabilize MTs from disassembly (Olmsted, 1986; Matus, 1988). Tau, microtubule-associated protein 2 (MAP2), and MAP4 are structurally related MAPs that have similar MT-binding domains but different projection domains that extend from the MT surface. These MAPs act as obstacles for MT motors in general and kinesin motors in particular and negatively influence their motility by decreasing the frequency of attachment to MTs and the average length of MT runs (Atherton et al., 2013). In contrast to tau/MAP2/MAP4, doublecortin and MAP7/ensconsin stimulate interaction of kinesin motors with MTs. Doublecortin and its homologue, doublecortin-like kinase 1, selectively enhance the affinity of the kinesin-3 Kif1A to MTs (Liu et al., 2012), and MAP7/ensconsin increases the frequency of productive encounters of the kinesin-1 motors with MTs (Sung et al., 2008; Metzger et al., 2012; Barlan et al., 2013a). The ability of MAPs to enhance or inhibit motility of specific kinesin motors makes them likely targets for signaling cascades that regulate the direction of MT transport. The recruitment of MAPs to MTs is controlled by phosphorylation, which negatively affects their ability to bind MTs (Lee, 1993; Avila et al., 1994; Mandell and Banker, 1996; Drewes et al., 1998; Cassimers and Spittle, 2001). Therefore phosphorylation-dependent changes in affinity of MAPs to MTs would be expected to affect the amount of plus-end-directed motility of kinesin cargoes and thus the overall direction of MT-based transport. However, whether MAPs regulate the direction of MT transport in cells has not been directly tested.

In this study, we examined the regulation of MT transport by MAPs using Xenopus melanophores as an experimental system. In these cells, thousands of membrane-bound pigment granules move along radial MTs to the cell center (pigment aggregation) or the periphery (pigment dispersion) by means of cytoplasmic dynein and kinesin-2, respectively (Nascimento and Kinne, 1996; Drewes et al., 1998; Cassimers and Spittle, 2001). Therefore phosphorylation-dependent changes in affinity of MAPs to MTs would be expected to affect the amount of plus-end-directed motility of kinesin cargoes and thus the overall direction of MT-based transport. However, whether MAPs regulate the direction of MT transport in cells has not been directly tested.

RESULTS

XMAP4 is phosphorylated during pigment aggregation

To gain insight into the regulation of pigment transport in melanophores and understand the role of MAPs in this regulation, we compared the phosphoproteomic profiles of cells stimulated to aggregate or disperse pigment granules. Phosphopeptides in unfractionated lysates of melanophores were enriched on iron immobilized metal ion affinity chromatography or with TiO2 resin. We identified >5000 unique phosphopeptides whose abundance increased in response to aggregation or dispersion signals. These peptides were derived from 2045 different proteins. Quantitative analysis of the phosphoproteomic data revealed 62 proteins whose phosphorylation levels changed in response to aggregation or dispersion stimuli more than fourfold. Among them were seven cytoskeleton-related proteins and only one structural MAP, XMAP4, whose phosphorylation increased during pigment aggregation. We cloned XMAP4 by PCR using cDNA synthesized from total RNA isolated from Xenopus melanophores as a template and a pair of primers specific to the published nucleotide sequence of XMAP4 from Xenopus oocytes. The amino acid sequence of the melanophore-specific XMAP4 was identical to the sequence of XMAP4 from oocytes, except for a deletion of 57 amino acid residues at the C-terminus and insertion of 10 amino acid residues in the middle of the molecule. We identified the amino acid residues phosphorylated during pigment aggregations as Thr-758 and Thr-762 located in the proline-rich region of the MT-binding domain (Figure 1). Phosphorylation of XMAP4 at Thr-758 and Thr-762 in melanophores stimulated to aggregate pigment increased more than fivefold compared with cells with dispersed pigment granules. Previous work showed that these threonines were targets of p34cdc2 and MAP kinases known to reduce the ability of mammalian MAP4 to bind MTs in HeLa cells (Ookata et al., 1995, 1997; Shinya and Tsukita, 1999; Kitazawa et al., 2000; Chang et al., 2001). Taken together, these results indicate that XMAP4 is phosphorylated in melanophores in response to pigment aggregation signals and suggest that this phosphorylation reduces the affinity of XMAP4 to MTs.

Overexpression of XMAP4 slows aggregation of pigment granules

Phosphorylation during pigment aggregation suggested that XMAP4 was directly or indirectly involved in the regulation of pigment granule transport. To understand the role of XMAP4 in transport of pigment granules, we examined the effect of XMAP4
overexpression on pigment aggregation and dispersion. XMAP4 was expressed in melanophores as an enhanced green fluorescent protein (EGFP) fusion to identify the overexpressing cells. Fluorescence microscopy indicated that, as expected, EGFP-XMAP4 bound cytoplasmic MTs (Supplemental Figure S1). To measure the effects of EGFP-XMAP4 overexpression on pigment granule transport, we stimulated pigment aggregation or dispersion and quantified the fractions of cells with aggregated, partially responded, or dispersed pigment granules 15 min after stimulation. Nontransfected melanophores or cells expressing EGFP were used as controls. We found that overexpression of EGFP-XMAP4 did not affect significantly pigment granule dispersion (Figure 2A) but dramatically inhibited their aggregation, as evidenced from the approximately eightfold and approximately twofold increases in the fractions of cells whose pigment granules remained completely and partially dispersed, respect-

Inhibition of pigment granule aggregation in cells overexpressing EGFP-XMAP4 is not explained by reduced rate of transfer of pigment granules from actin filaments onto MTs

Our data showed that overexpression of EGFP-XMAP4 dramatically delayed aggregation of pigment granules. Pigment aggregation includes two independent steps—the transfer of pigment granules from actin filaments onto MTs, and subsequent transport along MTs to the cell center. Either step could be inhibited by the overexpression of EGFP-XMAP4. Our previous work showed that the transfer step involves capture of pigment granules by growing MT tips and that the +TIP protein CLIP170 is essential for this process (Lomakin et al., 2009, 2011). It was therefore possible that EGFP-XMAP4 inhibited pigment granule capture by suppressing MT dynamics or preventing accumulation of CLIP-170 at the MT plus ends. In support of this possibility, overexpression of MAP4 in mammalian cells stabilized cytoplasmic MTs (Nguyen et al., 1997; Holmfeldt et al., 2002), and MAP1b, a MAP structurally related to MAP4, reduced the levels of MT-associated EB1, an adaptor protein that facilitates binding of CLIP-170 to MT tips (Tortosa et al., 2013). We therefore sought to determine whether overexpression of EGFP-XMAP4 inhibited pigment granule aggregation by suppressing MT dynamics or reducing binding of CLIP-170 to the plus ends of MTs.

To examine the effect of EGFP-XMAP4 overexpression on MT dynamics, we compared parameters of MT dynamic instability in melanophores induced to aggregate pigment granules overexpressing EGFP-XMAP4 and EGFP by injecting Cy3-tubulin into...
EGFP-positive cells, tracking tips of growing and shortening MTs, and decomposing tip trajectories into periods of growth, shortening, and pauses. We detected small but significant differences in the parameters of MT dynamic instability between the EGFP-XMAP4- and EGFP-expressing cells (Table 1). In particular, the average lengths of growth and shortening episodes were slightly reduced and the average duration of pause episodes increased in the melanophores overexpressing EGFP-XMAP4 (Table 1). To determine whether these changes affected the rate of pigment granule aggregation, we used a stochastic computational model for pigment aggregation that we previously developed (Lomakin et al., 2009, 2011). The model computes kinetics of pigment granule aggregation based on the statistics of bidirectional movement of pigment granules, the probability of pigment granule capture by growing MT tips, and parameters of MT dynamic instability and allows one to estimate independent effects of changes in MT dynamics on the pigment aggregation rate (Zaliapin et al., 2005; Lomakin et al., 2009, 2011). The output of the model is the kinetics of gray level decay, which reflects how fast the cytoplasm becomes increasingly transparent as pigment granules accumulate in the cell center. We first tested the model by performing experimental measurements of gray level decrease in cells expressing EGFP and comparing experimental data with the results of simulations that used the parameters of MT dynamic instability and pigment granule movement measured in these cells (Tables 1 and 2). This comparison showed a close match between the experimental and computed kinetic curves (Supplemental Figure S2). These results, in agreement with our previous analyses (Zaliapin et al., 2005; Lomakin et al., 2009, 2011), demonstrated that the computational model closely reproduced the kinetics of pigment aggregation. We next simulated aggregation of pigment granules using parameters of MT dynamic instability measured in melanophores overexpressing EGFP-XMAP4 (Table 1) without changing the granule movement statistics. These computer simulations generated kinetics of gray level decrease (Figure 3A, open squares) that was close to the kinetics measured for the EGFP-expressing cells (Figure 3A, open circles), with a half-time 4.36 ± 0.19 min. The experimentally measured half-time of gray level decrease estimated for melanophores overexpressing EGFP-XMAP4 (Figure 3A, black squares) was 27.78 ± 4.77 min, much longer than the half-time computed on the basis of dynamic instability parameters. Therefore changes in MT dynamics could not explain the dramatic inhibition of pigment aggregation observed in the EGFP-XMAP4–overexpressing cells.

To determine whether EGFP-XMAP4 displaced CLIP-170 from MT plus ends, we immunostained melanophores overexpressing EGFP or EGFP-XMAP4 with a CLIP-170 antibody and used images of the immunostained cells to generate normalized profiles of the CLIP-170 fluorescence at MT plus ends (Figure 3B). Quantitative analysis of the fluorescence profiles showed that the average length of MT segments decorated with CLIP-170 was similar between the EGFP- and EGFP-XMAP4–overexpressing melanophores (0.6 ± 0.05 and 0.68 ± 0.06 μm, respectively). Absolute values of CLIP-170 fluorescence per MT end were also similar between melanophores expressing EGFP and EGFP-XMAP4 (331 ± 13 and 428 ± 15 arbitrary units, respectively). Thus overexpression of EGFP-XMAP4 did not remove CLIP-170 from the MT plus ends. Taken together, the results of these experiments indicate that inhibition of pigment aggregation in melanophores overexpressing EGFP-XMAP4 is not explained by the reduced transfer of pigment granules from actin filaments onto MTs during pigment aggregation.

**Overexpression of EGFP-XMAP4 reduces the length of minus-end MT runs of pigment granules during pigment aggregation**

Our data indicated that overexpression of EGFP-XMAP4 did not affect capture of pigment granules by growing MT tips during pigment aggregation. During pigment aggregation, granules captured by MTs run to the MT minus ends focused in the cell center. We therefore tested whether overexpression of EGFP-XMAP4 affected minus end–directed granule motility by tracking individual pigment granules and decomposing motion trajectories into plus- and minus-end runs and pauses. For pigment granule tracking, we used melanophores expressing EGFP-XMAP4 at low levels, which aggregated pigment granules at a slightly reduced rate. This partial inhibition of pigment aggregation allowed us to estimate parameters of bidirectional granule movement. Control experiments involved tracking of pigment granules in melanophores expressing EGFP.

**TABLE 1:** Parameters of MT dynamic instability in melanophores with aggregated pigment expressing EGFP-XMAP4 or EGFP.

<table>
<thead>
<tr>
<th>Dynamic parameter</th>
<th>EGFP-XMAP4–expressing cells</th>
<th>EGFP-expressing cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth length (μm)</td>
<td>1.06 ± 0.09*</td>
<td>1.41 ± 0.10*</td>
</tr>
<tr>
<td>Growth rate (μm/s)</td>
<td>0.09 ± 0.01</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td>Shortening length (μm)</td>
<td>0.73 ± 0.09*</td>
<td>1.27 ± 0.11*</td>
</tr>
<tr>
<td>Shortening rate (μm/s)</td>
<td>0.09 ± 0.01*</td>
<td>0.13 ± 0.01*</td>
</tr>
<tr>
<td>Catastrophe frequency (s⁻¹)</td>
<td>0.048 ± 0.001*</td>
<td>0.041 ± 0.001*</td>
</tr>
<tr>
<td>Rescue frequency (s⁻¹)</td>
<td>0.053 ± 0.001*</td>
<td>0.047 ± 0.001*</td>
</tr>
<tr>
<td>Pause duration (s)</td>
<td>5.19 ± 0.31*</td>
<td>3.96 ± 0.22*</td>
</tr>
<tr>
<td>Number of analyzed MTs</td>
<td>40</td>
<td>41</td>
</tr>
<tr>
<td>Number of analyzed cells</td>
<td>20</td>
<td>14</td>
</tr>
</tbody>
</table>

Values are marked with an asterisk if the difference of the mean parameter value between EGFP-XMAP4– and EGFP-expressing cells is significant at the 0.05 level, according to a one-way analysis of variance test.

**TABLE 2:** Parameters of MT-based movement of pigment granules during pigment aggregation in melanophores expressing EGFP-XMAP4 or EGFP

<table>
<thead>
<tr>
<th>Movement parameter</th>
<th>EGFP-XMAP4–expressing cells</th>
<th>EGFP-expressing cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Velocity of minus-end runs (nm/s)</td>
<td>401.5 ± 7.5</td>
<td>386.6 ± 8.5</td>
</tr>
<tr>
<td>Length of minus-end runs (nm)</td>
<td>117.1 ± 4.5*</td>
<td>161.9 ± 8.6*</td>
</tr>
<tr>
<td>Velocity of plus-end runs (nm/s)</td>
<td>355.5 ± 9.2</td>
<td>337.9 ± 10.1</td>
</tr>
<tr>
<td>Length of plus-end runs (nm)</td>
<td>36.8 ± 0.7*</td>
<td>39.5 ± 1.2*</td>
</tr>
<tr>
<td>Duration of pauses (s)</td>
<td>0.69 ± 0.03</td>
<td>0.66 ± 0.03</td>
</tr>
<tr>
<td>Number of analyzed pigment granules</td>
<td>121</td>
<td>80</td>
</tr>
</tbody>
</table>

Values are marked with an asterisk if the difference of the mean parameter value between EGFP-XMAP4– and EGFP-expressing cells is significant at the 0.05 level, according to a one-way analysis of variance test.
Removal of XMAP4 from MTs selectively inhibits dispersion of pigment granules

To further understand the role of XMAP4 in pigment transport, we next tested whether removal of XMAP4 from MTs affected aggregation or dispersion of pigment granules. To remove XMAP4 from MTs, we produced antibodies against the XMAP4 microtubule-binding domain (MBD). Such antibodies have been shown to block binding of mammalian MAP4 to MTs in vivo and in vitro (Wang et al., 1996). We performed several control experiments to confirm that our antibodies against XMAP4 MBD were specific and had the predicted blocking effect.

To examine whether MBD antibodies were specific for XMAP4, we performed immunoblotting with whole-cell extracts. In extracts of control, nontransfected melanophores the antibodies recognized a major band with apparent molecular weight ∼250 kDa (Figure 4A, left). The additional lower–molecular weight bands bound to MBD antibodies (Figure 4A) likely represented degradation products of XMAP4, since the corresponding proteins cosedimented with MTs assembled in cell extracts (unpublished data). In extracts of melanophores overexpressing EGFP-XMAP4, MBD antibodies recognized an additional band with electrophoretic mobility characteristic of the XMAP4 (Figure 4A, right). The apparent molecular weights of the immunoreactive proteins were higher than the values predicted for the XMAP4 or GFP-XMAP4 based on the amino acid sequence. However, previous studies showed that the mobility of mammalian MAP4 on SDS gels was also unusually slow (Aizawa et al., 1990; West et al., 1991; Chapin et al., 1995). Therefore immunoblotting experiments showed that MBD antibodies were specific for the XMAP4.

To determine whether MBD antibodies removed XMAP4 from MTs in vitro, we transfected MTs in cell extracts in the presence or absence of control nonimmune immunoglobulin G (IgG) or MBD antibodies, pelleted MTs through a glycerol cushion to separate soluble and MT-bound MAPs, and examined the composition of MAPs in the MT pellets. MTs assembled in cell extracts bound numerous MAPs (Figure 4B, left), and preincubation of extracts with nonimmune IgG did not change composition of MAPs in the MT pellets. However, preincubation with MBD antibodies removed from MT pellets a single band with electrophoretic mobility characteristic of XMAP4 (Figure 4B, right). Western blots confirmed that XMAP4 was absent from the pellets of MTs assembled in the presence of MBD antibodies but not control nonimmune IgG (Supplemental Figure S3). Therefore MBD antibodies completely and selectively blocked the binding of XMAP4 to MTs in vitro.

Comparison of pigment granule movement parameters between populations of melanophores expressing EGFP-XMAP4 and EGFP (Supplemental Table S1). We conclude that expression of EGFP-XMAP4 does not significantly affect parameters of MT dynamic instability or loss of CLIP-170 from the MT tips. (A) Comparison of kinetics of CLIP-170 fluorescence at MT plus ends normalized by maximum fluorescence and averaged for melanophores expressing EGFP (black squares) or EGFP-XMAP4 (white circles); expression of EGFP-XMAP4 does not significantly change the distribution of the CLIP-170 fluorescence at the MT plus ends.
These spots likely represented EGFP-XMAP4–MBD antibody complexes. Microinjection of control nonimmune IgG did not affect MT localization of EGFP-XMAP4 (unpublished data), which indicated that the blocking effect of the MBD antibodies was highly specific. Immunofluorescence staining with anti-tubulin antibody showed that the MT network appeared normal in the MBD antibody–injected cells (Supplemental Figure S4). Furthermore, antibody microinjection did not significantly affect parameters of MT dynamic instability. The lengths of growth and shortening episodes were slightly reduced in antibody-injected cells compared with melanophores injected with nonimmune IgG (Supplemental Table S2). However, growth and shortening of MTs were affected to about the same extent, and therefore these changes in MT dynamics could not cause significant reduction in MT density. These results are in line with published data that showed that depleting MAP4 from MTs had no detectable effect on the distribution or dynamics of MTs in human fibroblasts and monkey kidney epithelial cells (Wang et al., 1996). Therefore microinjection of antibodies against MBD into melanophores abolished binding of XMAP4 to MTs without changing MT organization or dynamics.

We next tested whether removal of XMAP4 from MTs affected pigment granule transport. We injected melanophores with MBD antibodies or nonimmune IgG, stimulated pigment aggregation or dispersion, and estimated the fractions of cells with aggregated, partially responded, or dispersed pigment granules. Microinjection of antibodies against MBD significantly inhibited pigment dispersion, as evidenced by the approximately ninefold increase in the fraction of cells whose response to dispersion signal was completely inhibited and a twofold increase in the fraction of cells that showed partial inhibition compared with melanophores microinjected with nonimmune IgG. However, growth and shortening of MTs were affected to about the same extent, and therefore these changes in MT dynamics could not cause significant reduction in MT density. These results are in line with published data that showed that depleting MAP4 from MTs had no detectable effect on the distribution or dynamics of MTs in human fibroblasts and monkey kidney epithelial cells (Wang et al., 1996). Therefore microinjection of antibodies against MBD into melanophores abolished binding of XMAP4 to MTs without changing MT organization or dynamics.

We next tested whether removal of XMAP4 from MTs affected pigment granule transport. We injected melanophores with MBD antibodies or nonimmune IgG, stimulated pigment aggregation or dispersion, and estimated the fractions of cells with aggregated, partially responded, or dispersed pigment granules. Microinjection of antibodies against MBD significantly inhibited pigment dispersion, as evidenced by the approximately ninefold increase in the fraction of cells whose response to dispersion signal was completely inhibited and a twofold increase in the fraction of cells that showed partial inhibition compared with melanophores microinjected with nonimmune IgG.

**FIGURE 4:** Displacement of XMAP4 from MTs by injection of MBD antibodies inhibits dispersion but not aggregation of pigment granules. (A) Immunoblotting with MBD antibodies of whole-cell extracts of control nontransfected cells (left) or melanophores overexpressing EGFP-XMAP4 (right); MBD antibodies recognize the XMAP4 band in whole-cell extracts of control cells and an additional EGFP-XMAP4 band in whole-cell extracts of EGFP-XMAP4–overexpressing cells. (B) Coomassie-stained SDS gels of pelleted MTs assembled in whole-cell extracts preincubated without added IgG (left), in the presence of control nonimmune IgG (middle), or antibodies against XMAP4 MBD (right); preincubation of whole-cell extracts with MBD antibodies prevents cosedimentation of XMAP4 but not other MAPs with MTs. (C) Live images of a melanophore expressing EGFP-XMAP4 before (left) and 30 min after (right) injection of antibodies against XMAP4 MBD; scale bar, 20 μm; the antibody injection completely removes XMAP4 from the MTs. (D) Quantification of response to dispersion (left) or aggregation (right) stimuli of melanophores microinjected with nonimmune IgG or antibodies against XMAP4 MBD. Microinjection of MBD antibodies does not significantly affect pigment aggregation but markedly inhibits pigment dispersion, as evidenced by increases in the fractions of cells with aggregated or partially responded pigment granules compared with melanophores microinjected with nonimmune IgG.
with nonimmune IgG (Figure 4D, left). Aggregation of pigment granules was not significantly affected, given that the fractions of cells with completely or partially aggregated pigment granules were similar between populations of melanophores microinjected with MBD antibodies or nonimmune IgG (Figure 4D, right). Furthermore, microinjection of MBD antibodies did not significantly change parameters of bidirectional granule movement during pigment aggregation (Supplemental Table S3). Thus removal of XMAP4 from MTs selectively inhibited dispersion of pigment granules.

Dispersion of pigment granules in XMAP4 antibody–injected cells could be inhibited through decrease in the length or velocity of plus-end granule runs, increase in the length or velocity of minus-end runs, or both. To determine how depletion of XMAP4 from MTs affected parameters of bidirectional granule movement, we microinjected cells with MBD antibodies or control nonimmune IgG and recorded and tracked individual pigment granules shortly after the application of a pigment dispersion stimulus. To allow for the tracking of pigment granules, we decreased the amounts of MBD antibodies and nonimmune IgG delivered into the cytoplasm during microinjection by reducing microinjection time by ~50%. Reduced cytoplasmic levels of MBD antibodies slowed pigment dispersion but did not block it completely, as evidenced by the increased fraction of cells with partially dispersed pigment granules compared with melanophores injected with control nonimmune IgG (Supplemental Figure S5). Partial inhibition of pigment granule dispersion made possible measurements of the velocity and length of bidirectional granule runs. Comparison of granule movement statistics between cells microinjected with MBD antibodies and control IgG showed that MBD antibodies reduced pigment granule runs in both directions and that the average length of runs was affected more significantly than run velocity, reaching ~70% of the control values (Table 3). Further, the average length of plus- and minus-end runs was reduced to about the same extent, but the net effect was more prominent in the case of longer plus-end runs, which shortened by ~30 nm (Table 3). We conclude that XMAP4 positively regulates motility of pigment granules along MTs in both directions and stimulates pigment dispersion by increasing the length of plus-end granule runs.

<table>
<thead>
<tr>
<th>Movement parameter</th>
<th>XMAP4 antibody–injected cells</th>
<th>Control IgG-injected cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Velocity of minus-end runs (nm/s)</td>
<td>250.3 ± 9.9</td>
<td>272.0 ± 10.1</td>
</tr>
<tr>
<td>Length of minus-end runs (nm)</td>
<td>53.8 ± 1.5*</td>
<td>74.4 ± 4.1*</td>
</tr>
<tr>
<td>Velocity of plus-end runs (nm)</td>
<td>250.7 ± 7.5</td>
<td>271.2 ± 7.6</td>
</tr>
<tr>
<td>Length of plus-end runs (nm)</td>
<td>111.3 ± 4.1*</td>
<td>141.5 ± 5.5*</td>
</tr>
<tr>
<td>Duration of pauses (s)</td>
<td>1.1 ± 0.1</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>Number of analyzed pigment granules</td>
<td>98</td>
<td>116</td>
</tr>
</tbody>
</table>

Values are marked with an asterisk if the difference of the mean parameter value between XMAP4 antibody and IgG-injected cells is significant at the 0.05 level, according to a one-way analysis of variance test.

TABLE 3: Parameters of MT-based movement of pigment granules during pigment dispersion in melanophores microinjected with antibody against XMAP4 MT-binding domain or control IgG.
binding of mammalian MAP4 to MTs (Illenberger et al., 1996; Kitazawa et al., 2000; Chang et al., 2001). Furthermore, threonine residues in the mammalian MAP4 that correspond to the XMAP4Thr-758 and Thr-762 are phosphorylated by p34**cdc2** and MAP protein kinases during the transition from interphase to mitosis, and this phosphorylation correlates with a decrease in the ability of MAP4 to bind cytoplasmic MTs (Ookata et al., 1995, 1997; Shina and Tsukita, 1999). We hypothesized that phosphorylation of XMAP4 at Thr-758 and Thr-762 reduces the binding of XMAP4 to MTs, removing physical barriers in the path of dynein motors moving pigment granules to the cell center, thus accelerating pigment aggregation.

To determine whether phosphorylation of XMAP4 at Thr-758 and Thr-762 affects aggregation of pigment granules, we compared the response to aggregation signal of melanophores overexpressing the EGFP-tagged Thr-758E/Thr-756E phosphomimetic or Thr-758A/Thr-756A nonphosphorylatable XMAP4 mutants. Measurement of pigment aggregation responses showed that the phosphomimetic XMAP4 mutant had a reduced inhibitory effect on pigment aggregation compared with the nonphosphorylatable mutant, as evidenced by the statistically significant (p = 0.03) increase in the fraction of cells with aggregated pigment granules from 29 to 41%, concomitant with a decrease in the fraction of melanophores with dispersed pigment (Figure 6A). This effect could not be explained by a difference in the expression levels of mutant proteins (Figure 6B). As expected, overexpression of phosphomimetic or nonphosphorylatable XMAP4 mutants did not significantly affect pigment dispersion (Supplemental Figure S6). Therefore phosphorylation at Thr-758 and Thr-756 reduced the inhibitory effect of XMAP4 on pigment aggregation.

To test whether phosphorylation at Thr-758 and Thr-756 affected binding of XMAP4 to MTs, we expressed hexahistidine (6xHis)-

tagged phosphomimetic and nonphosphorylatable mutants in baculovirus, purified recombinant proteins by chromatography on Ni-nitrilotriacetic acid (NTA) agarose (Supplemental Figure S7), and compared their ability to cosediment with MTs in vitro.

For the MT cosedimentation, we combined purified XMAP4 phosphomimetic or nonphosphorylatable mutant proteins with p-clitaxel-stabilized MTs to attain a final concentration equimolar to tubulin and pelleted MTs by high-speed centrifugation. We found that the Thr-758E/Thr-756E band was reduced in the MT pellets and increased in the supernatants compared with the Thr-758A/Thr-756A band (Figure 6B, top). Measurement the amounts of XMAP4 mutant proteins in the MT pellets using quantitative immunoblotting confirmed that intensity of the Thr-758E/Thr-756E band was on average ~1.4-fold lower than that of the Thr-758A/Thr-756A band (Figure 6B). Therefore binding to MTs of the phosphomimetic mutant protein compared with the nonphosphorylatable mutant protein was significantly decreased. We conclude that phosphorylation at Thr-758 and Thr-756 reduces binding of XMAP4 to MTs and partially relieves inhibition of pigment aggregation in the overexpressing cells.

Our phosphoproteomic profiling indicated that phosphorylation of XMAP4 is increased during pigment granule aggregation, and the results of MT cosedimentation experiments showed that this phosphorylation reduced binding of XMAP4 to MTs. Therefore the levels of endogenous MT-bound XMAP4 should decrease during pigment aggregation. We therefore compared the levels of MT-bound XMAP4 in melanophores stimulated to aggregate or disperse pigment granules by measuring XMAP4 fluorescence after immunostaining with antibodies against XMAP4 MBD. To reduce background fluorescence, we extracted cells with 0.5% Triton X-100 before fixation. We found that the endogenous XMAP4 was associated with MTs in both signaling states. However, XMAP4 MT fluorescence in cells stimulated to disperse pigment granules appeared brighter than in melanophores induced to disperse pigment granules (Supplemental Figure S8). Averaging measurements of XMAP4 MT fluorescence in 20 cells in each signaling state confirmed that the brightness of MTs was higher in cells treated to disperse than aggregate pigment granules (60.9 ± 1.39 vs. 45.6 ± 1.44 arbitrary units, respectively; mean ± SEM; n = 100). This result was highly statistically significant (p = 1.4 × 10^-12). We conclude that pigment aggregation signals that induce XMAP4 phosphorylation partially remove XMAP4 from MTs.

DISCUSSION

In this study, we identified XMAP4 as an important regulator of MT-based transport of pigment granules in Xenopus melanophores. This conclusion is based on several lines of experimental evidence. First, overexpression of XMAP4 significantly slowed pigment aggregation, without affecting pigment dispersion. This effect could be explained by a reduced length of minus-end MT runs of pigment granules in overexpressing cells. Second, removal of XMAP4 from MTs did not influence pigment aggregation but suppressed pigment dispersion by reducing the length of plus-end granule runs. Finally, binding of XMAP4 to MTs and the inhibition of pigment granule aggregation by overexpression of XMAP4 were reduced by using phosphomimetic mutations. Therefore our work for the first time demonstrates that phosphorylation-dependent changes in the binding of a structural MAP to MTs regulate the direction of MT transport of membrane organelles.

Our data show that overexpression of XMAP4 reduces the average length of minus-end MT runs of pigment granules generated by dynein. This result is in line with the results of other studies that indicate that MAP4 inhibits dynein motility. Overexpression of MAP4 in
interphase mammalian cells prevents clustering of the Golgi cisternae in the cell center, which requires dynein activity (Bulinski et al., 1997). Similarly, MAP4 suppresses dynein-dependent sliding of astral MTs at the cell cortex essential for positioning of the mitotic spindle and inhibits gliding of MTs powered by dynein in vitro (Samora et al., 2011). On the other hand, motility of single dynein molecules along MTs in vitro was relatively insensitive to decoration with nonphosphorylatable or phosphomimetic XMAP4 (Dixit et al., 2008; Vershinin et al., 2011). On the other hand, motility of single dynein molecules along MTs in vitro was relatively insensitive to decoration with nonphosphorylatable or phosphomimetic XMAP4 (Dixit et al., 2008; Vershinin et al., 2011). However, in living cells, dynein motors moving pigment granules have to overcome significant viscous drag and therefore might be more susceptible to obstacles on the MT surface. Further, XMAP4 might be a more efficient inhibitor of dynein motility than MT-binding fragments of tau used in vitro motility assays. Therefore it is likely that XMAP4 inhibits minus end–directed MT movement of pigment granules during pigment aggregation by blocking the path of dynein motors.

In a marked contrast to pigment aggregation, overexpression of XMAP4 does not inhibit dispersion of pigment granules, which requires the activity of kinesin-2 (Tuma et al., 1998). Thus, unlike dynein, kinesin-2 is capable of moving along MTs bearing XMAP4. The lack of effect on kinesin-2–driven transport might be explained by the particular isoform of XMAP4 expressing in Xenopus melanophores. Only the five-repeat isoform, and not the three- or four-repeat isoforms, of mammalian MAP4 suppresses gliding MTs driven by kinesin-1 in vitro motility assays (Tokuraku et al., 2007), and it is possible that kinesin-2 shares with kinesin-1 insensitivity to the MAP4 isoforms with reduced numbers of MT-binding repeats. Therefore specific isoforms of XMAP4 might selectively affect motility of MT motors similar to yeast protein She1, which inhibits movement of dynein but not kinesins along MT tracks (Markus et al., 2012). However, it is also possible that movement along MTs with bound XMAP4 or other MAPs is an inherent property of kinesin-2, as the unique neck-linker region allows kinesin-2 to navigate obstacles on the MT surface (Hoeprich et al., 2014). Unlike kinesin-1 or cytoplasmic dynein, which take straight paths, kinesin-2 family members are capable of switching protofilaments (Brunnbauer et al., 2012). The sideways steps may let kinesin-2 bypass molecules of XMAP4 and other MAPs extending from the MT wall (Hoeprich et al., 2014).

We unexpectedly discovered that XMAP4 positively regulates plus–end–directed runs of pigment granules during pigment dispersion. This conclusion is based on the results of experiments that involved microinjection of blocking antibodies that removed XMAP4 from MTs. In Xenopus melanophores, plus-end runs of pigment granules have been shown to be generated by kinesin-2 (Tuma et al., 1998). Therefore our data suggest that, similar to MAP7/ensconsin, which activates kinesin-1 (Sung et al., 2008; Metzger et al., 2012; Barlan et al., 2013a), and doublecortin/doublecortin-like kinase, which facilitates transport of membrane vesicles driven by Kif1A (Liu et al., 2012), XMAP4 stimulates kinesin-2 motility. Doublecortin increases the length of MT runs of Kif1A cargoes by enhancing binding of the Kif1A motor domain to MTs (Liu et al., 2012). We hypothesize that XMAP4 might stimulate kinesin-2 motility by a similar mechanism. Our pull-down experiments indicate that, unlike doublecortin, which forms a complex with Kif1A, XMAP4 does not binds kinesin-2 directly. However, we instead observe an interaction between XMAP4 and the p150Glued subunit of the dynactin complex, an adaptor to MT motor proteins on the granule surface (Deacon et al., 2003). Mammalian MAP4 also immunoprecipitates p150Glued minus end–directed MT movement of pigment granules during pigment aggregation by blocking the path of dynein motors.

FIGURE 6: Phosphorylation of XMAP4 reduces inhibition of pigment aggregation and decreases binding to MTs. (A) Top, quantification of the response to a pigment aggregation stimulus of melanophores overexpressing nonphosphorylatable (left set of bars) or phosphomimetic (right set of bars) mutants or XMAP4; data are expressed as the percentages of cells with aggregated (white bars), partially responded (gray bars), or dispersed (black bars) pigment granules. Bottom, comparison of the levels of expression of XMAP4 mutants based on the EGFP fluorescence. Overexpression of the phosphomimetic XMAP4 mutant has a weaker inhibitory effect compared with the nonphosphorylatable mutant, as evidenced by a smaller fraction of cells with dispersed and larger fraction of cells with aggregated pigment granules compared with melanosomes overexpressing the XMAP4 nonphosphorylatable mutant; this difference cannot be explained by the reduced expression levels of the phosphomimetic mutant, given that the mutants are expressed at approximately the same levels. (B) Cosedimentation of recombinant nonphosphorylatable and phosphomimetic XMAP4 mutant proteins with MTs in vitro. Top, Coomassie-stained gel, which shows protein composition of the input (XMAP4 mutant protein–MT mixtures before centrifugation; I), supernatants (S), and MT pellets (P). Bottom, measurement of relative amounts of nonphosphorylatable (left) and phosphomimetic (right) mutant proteins in MT pellets using quantitative immunoblotting; the data represent averages for the values measured in three independent experiments; inset, representative images of the XMAP4 mutant bands. The amount of the phosphomimetic mutant protein in the MT pellets is significantly smaller, and in the supernatants larger, than the nonphosphorylatable mutant, which indicates reduced binding to MTs.
Phosphorylation during pigment aggregation reduces binding of XMAP4 to MTs, thus increasing minus end–directed and decreasing plus end–directed motility of pigment granules (left), which stimulates their accumulation at the cell center, whereas dephosphorylation of XMAP4 during pigment dispersion has an opposite effect (right).

Surprisingly, our data indicate that effects of XMAP4 on granule runs in each direction are different in the two signaling states. Overexpression of XMAP4 does not increase the average length of plus-end runs during pigment granule aggregation as would be expected in light of a positive effect of XMAP4 on plus-end run length during dispersion of pigment granules. Similarly, removal of XMAP4 from MTs during granule dispersion reduced the length of minus-end granule runs, which is apparently inconsistent with the minus-end run length decrease during granule aggregation in XMAP4-overexpressing cells. We hypothesize that the observed differences in effects of XMAP4 on granule runs during pigment aggregation and dispersion might be explained by variations in sensitivity to XMAP4 by the active and inactive states of MT motors. In the fully active state typical for pigment aggregation, dynein might be highly sensitive to XMAP4 obstacles on the MT surface. On the contrary, an inactive state characteristic of pigment dispersion, dynein processivity is enhanced by XMAP4, presumably through interaction of XMAP4 with p150Glued on the granule surface. Similarly, XMAP4 enhances plus end–directed granule runs generated by active kinesin-2 motors during pigment dispersion, but binding of XMAP4 to MTs might be insufficient to activate kinesin-2 motility during aggregation of pigment granules. It is therefore likely that reversible binding of XMAP4 to MTs cooperates with independent regulation of the activities of MT motors to control the direction of MT-based transport of pigment granules.

FIGURE 7: Hypothesis for the regulation of MT-based transport of pigment granules by XMAP4. XMAP4 negatively regulates minus end–directed MT transport of pigment granules by blocking the movement of dynein motors along MTs and positively regulates plus end–directed, kinesin-2–based transport through interaction with the granule-bound p150Glued. This interaction increases processivity of kinesin-2 motors by keeping them in proximity to the MT surface. Phosphorylation during pigment aggregation reduces binding of XMAP4 to MTs, thus increasing minus end–directed and decreasing plus end–directed motility of pigment granules (left), which stimulates their accumulation at the cell center, whereas dephosphorylation of XMAP4 during pigment dispersion has an opposite effect (right).
MATERIALS AND METHODS

Cell culture

Xenopus melanophores (Kashina et al., 2004) were cultured in Xenopus tissue culture medium (70% L15 medium supplemented with 0.2 mg/ml penicillin, 0.2 mg/ml streptomycin, 5 μg/ml insulin, and 10% heat-inactivated fetal bovine serum) at 27°C. Before stimulation of aggregation or dispersion, melanophores were transferred into serum-free 70% L15 medium and incubated for at least 1 h at 27°C. Aggregation of pigment granules was induced by treatment with 10−8 M melatonin. For dispersion of pigment granules, cells were treated with 10−8 M melanocyte-stimulating hormone (MSH).

Quanification of aggregation and dispersion responses of melanophores

To determine the fractions of cells with aggregated, partially responded, or dispersed pigment, melanophores were treated with melatonin or MSH for 15 min or 1 h and fixed with formaldehyde. The numbers of cells with aggregated, partially responded, or dispersed pigment granules were determined by counting cells in each category by phase-contrast microscopy as described previously (Kashina et al., 2004).

To determine kinetics of pigment aggregation, time series of bright-field images of melanophores treated with melatonin were acquired with 10-s time intervals. Integrated pixel values within cell outlines were determined for each of the acquired images using the region measurement tool of MetaMorph image acquisition and analysis software (Molecular Devices, Downingtown, PA). The percentage of gray levels was calculated for each image as described previously (Lomakin et al., 2011). Data were averaged across the recorded cells and plotted as a function of time.

Mass spectrometry

Melanophores grown in dense monolayers in two 75-cm² flasks were transferred into 70% serum-free L15 medium for 60 min, stimulated with either melatonin or MSH for 60 min, washed twice with 10 ml of ice-cold 0.7x phosphate-buffered saline (PBS), and lysed on ice by adding 1 ml of lysis buffer containing 8 M urea, 75 mM NaCl, 10 mM sodium pyrophosphate, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Cell lysates were sonicated and clarified by centrifugation at 100,000 g for 10 min at 4°C and washed with PBS. Cell pellets were resuspended in the lysis buffer (20 mM Tris, 500 mM NaCl, 10 mM Tris, pH 8.2, one tablet of protease inhibitor cocktail (Roche Applied Sciences, Indianapolis, IN) per 10 ml of lysis buffer, 1 mM NaF, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Cell lysates were sonicated and clarified by centrifugation. Proteins were reduced and alkylated and digested with trypsin as described previously (Villen and Gygi, 2008). After desalting and strong cation exchange (SCX) chromatography, phosphopeptides were selectively enriched by a combination of SCX chromatography and immobilized metal affinity chromatography (Villen and Gygi, 2008). Liquid chromatography–tandem mass spectrometry (MS/MS) was performed on an LTQ Orbitrap hybrid mass spectrometer (Thermo Fisher, San Jose, CA). MS/MS spectra were searched through the SEQUEST algorithm with the target-decoy strategy (Elias and Gygi, 2007) against a search via the SEQEST algorithm with the target-decoy strategy (Beausoleil et al., 2006) was used to assign phosphorylation site localization.

XMAP4 cloning and mutagenesis

DNA for the XMAP4 from Xenopus melanophores was amplified by PCR using a as template cDNA synthesized with SuperScriptII reverse transcriptase (Life Technologies, Carlsbad, CA) from the total melanophore RNA. The following set of primers designed against the X. laevis XMAP4 (National Center for Biotechnology Information accession number NP_001083770.1) was used for the DNA amplification: CCGGCTCGAGCATGGCGGACCTTGAGC (forward primer) and GCGGCCCCGGGTAGTGTGCTCTCTGG (reverse primer). The PCR product was cloned into mEGFP-C2 vector (Clontech, Mountain View, CA) using XhoI and SacII restriction sites and verified by sequencing. Amino acid sequence of the cloned protein was identical to the sequence published for XMAP4 except that it had a 10–amino acid insert (AEVLSAPIPE) between the amino acid residues 63 and 73 and a 57–amino acid deletion between the amino acid residues 1056–1112 in the C-terminal region of the molecule. Several independent amplification experiments produced PCR products with the same nucleotide sequence, which indicated that the amplified DNA encoded the principal isoform of XMAP4 expressed in melanophores.

Phosphomimetic (T758E/T762E) and nonphosphorylatable (T758A/T762A) mutants of XMAP4 were generated using QuikChange XL Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA).

Expression of XMAP4 mutant proteins in baculovirus

Recombinant phosphomimetic and nonphosphorylatable XMAP4 mutant proteins were expressed in baculovirus using the Bac-to-Bac Baculovirus expression system (Life Technologies). To facilitate purification of recombinant proteins and enable detection of protein expression, 6xHis and mCherry tags were added to the N-termini of the XMAP4 sequences. 6xHis/mCherry tag DNA was amplified by PCR using mCherry N1 mammalian expression vector (Clontech) as a template and cloned into pFastBac1 vector (Life Technologies) using BamHI and EcoRI restriction sites. PCR-amplified XMAP4 mutant DNAs were cloned into pFastBac1 vector downstream of the 6xHis/mCherry tag using SphI and XhoI restriction sites. Recombinant bacmid and baculovirus stocks were produced according to instructions provided by the kit manufacturer. For protein expression, Sf9 cells were infected with baculovirus and incubated for 48 h in SF-900 II medium (Life Technologies) containing 50 U/ml penicillin and 50 μg/ml streptomycin. Cells were pelleted at 1000 × g for 10 min at 4°C and washed with PBS. Cell pellets were resuspended in the lysis buffer (20 mM Tris, 500 mM NaCl, 10 mM imidazole, 1 mM MgCl2, 1% Triton X-100, 5 mM β-mercaptoethanol, 0.5 mM MgATP, 1 mM PMSF, pH 7.5) and incubated on ice for 45 min for the cell lysis. Cell lysates were clarified by centrifugation at 100,000 × g for 30 min at 4°C and used for purification of recombinant proteins by chromatography on Ni-NTA agarose (Qiagen, Valencia, CA) according to instructions provided by the manufacturer.

Cell transfection

Melanophores were transfected using GeneCellin DNA transfection reagent (Bulldog Bio, Portsmouth, NH) according to instructions provided by the manufacturer. After transfection, cells were incubated for 2–4 d at 27°C for protein expression.

Production of antibodies against XMAP4

For production of antibodies against the MBD of XMAP4, a fragment of DNA that encoded amino acids 722–1228 of XMAP4 was amplified by PCR using GGAATTCATTGACAAGACT C GCTG CAGC as forward primer and CCGGCTCGAGTTA GATGCT TGT TCTCTG TAGTA as reverse primer. The PCR product was cloned into the bacterial expression vector pCold I DNA (Clontech) using the Nde and XhoI restriction sites. Recombinant protein was expressed in Escherichia coli according to instructions provided by the vector manufacturer, purified by affinity chromatography on Ni-NTA agarose (Qiagen), dialyzed against PBS, and used for rabbit immunization. High-titer antisera were produced by Bio-Synthesis.
Polyclonal antibodies were purified from antisera by affinity chromatography on a CNBr-activated Sepharose 4B (Sigma-Aldrich, St. Louis, MO) with covalently attached antigen. Purified antibodies were dialyzed against microinjection buffer and concentrated by ultrafiltration.

Polyclonal antibodies against XMAP4 projection domain were generated by immunization of rabbits with the synthetic peptide GCDDDDVKEPKNSERASAAPHD manufactured by Bio-Synthesis, which corresponded to the amino acid residues 56–75 of XMAP4. Antibodies were purified by affinity chromatography of antisera on SulfoLink resin (Thermo Scientific, Rockford, IL) with covalently attached peptide antigen.

Cosedimentation of recombinant XMAP4 mutant proteins with MTs
MTs were assembled by polymerization of purified porcine brain tubulin (Cytskeleton, Denver, CO) in the presence of paclitaxel (20 μM; Sigma-Aldrich), and recombinant XMAP4 mutant proteins were added at 1:1 molar ratio to tubulin. After incubation for 30 min at room temperature, MTs were pelleted by centrifugation through a glycerol cushion at 140,000 x g for 40 min at 25°C, and the MT pellets were resuspended in SDS–PAGE sample buffer. The amounts of XMAP4 mutant proteins in the MT pellets were measured by quantitative immunoblotting.

Immunoprecipitation and immunoblotting
For immunoprecipitation, whole-cell extracts were incubated for 2 h at room temperature with rabbit affinity-purified anti-GFP antibodies (ab2290; Abcam, Cambridge, MA) or antibodies against XMAP4 MBD bound to protein A agarose (Thermo Scientific). Agarose beads were pelleted and washed, and adsorbed proteins were eluted by the treatment of beads with SDS–PAGE sample buffer for 5 min at 100°C.

Immunoblotting was performed by the method described in Towbin et al. (1979). Blots were stained with mouse monoclonal GFP antibodies (ab1218; Abcam), p150Glued subunit of the dynactin complex (610473; BD Biosciences, San Jose, CA), cytoplasmic dynein IC (74.1; Covance, Princeton, NJ), or kinesin-2 motor subunit (K2.4; Covance). Immunoreactive bands were detected with SuperSignal West Femto maximum-sensitivity substrate (Thermo Scientific, Waltham, MA). To measure the amounts of recombinant XMAP4, nonphosphorylatable and phosphomimetic mutant proteins cosedimented with MTs, blots of MT pellets were successively incubated with antibodies against XMAP4 MBD and IRDye800-conjugated affinity-purified anti-mouse IgG (Rockland Immunochemicals, Gilbertsville, PA), and the intensity of the infrared signal was quantified with the Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE).

Microinjection
Pressure microinjection of antibodies against XMAP4 MBD (8–10 mg/ml), nonimmune rabbit IgG (12 mg/ml), and Cy3-labeled porcine brain tubulin (6–7 mg/ml) was performed using the PLI-100 Pico-Liter microinjection system (Harvard Apparatus, Holliston, MA) as described previously (Semenova and Rodionov, 2007; Ikeda et al., 2010).

Immunofluorescence staining
For immunofluorescence staining of MTs, melanophores microinjected with antibody against XMAP4 MBD were briefly rinsed with PBS, fixed with glutaraldehyde, permeabilized with PBS containing 1% Triton X-100 (Ted Pella, Redding, CA), and sequentially incubated with mouse monoclonal antibodies against α-tubulin (1:200, DM1A; Cedarlane Laboratories, Burlington, NC) and goat anti-mouse antibodies conjugated with Alexa Fluor 488 (1:200; Invitrogen). Immunofluorescence staining of MTs was followed by the incubation cells with goat anti-rabbit antibodies conjugated with rhodamine (1:200; KPL, Gaithersburg, MD), which allowed us to identify the microinjected cells. For immunofluorescence staining of CLIP170 comets, melanophores were fixed in cold methanol, postfixed with 4% formaldehyde, permeabilized with 0.1% Triton X-100, and immunostained with antibodies specific for CLIP-170 N-terminus (no. 2221; 1:200; Hoogenraad et al., 2000) and goat anti-rabbit antibodies conjugated with Alexa 488 (1:200; Invitrogen).

For double immunofluorescence staining with XMAP4 MBD and tubulin antibodies, melanophores treated for 5 min with melatonin or 3 min with MSH were briefly rinsed with PBS and placed for 2 min in permeabilization buffer containing 60 mM 1,4-piperazinediethanesulfonic acid, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 10 mM ethylene glycol tetraacetic acid, 2 mM MgCl2, 20 mM paclitaxel, and 0.5% Triton X-100. After permeabilization, cells were washed with PBS, fixed with 1% glutaraldehyde for 30 min, treated with NaBH4 to quench the unreacted glutaraldehyde, and blocked with 2% solution of bovine serum albumin. Cells were then sequentially incubated with affinity-purified antibodies against XMAP4 MBD (0.7 μg/ml), goat anti-rabbit antibodies conjugated with rhodamine (1:200; KPL), mouse monoclonal antibodies against α-tubulin (1:200, DM1A; Cedarlane Laboratories), and goat-anti-mouse antibodies conjugated with Alexa Fluor 488 (1:200, Invitrogen). Immunostained cells were mounted in Aqua-Poly/Mount medium (Polysciences, Warrington, PA).

Image acquisition and analysis
Fluorescence images of melanophores were acquired using a Nikon Eclipse Ti inverted microscope equipped with a Plan Apochromat 100×/1.4 numerical aperture objective lens and Andor iXon EM-CCD sensor (Andor Technology, Windsor, CT) driven by MetaMorph image acquisition and analysis software (Molecular Devices, Downingtown, PA). MT dynamics was measured by acquiring time series of images of Cy3-labeled MTs, as described previously (Semenova and Rodionov, 2007). To reduce photobleaching and photodamage, cells were treated with the oxygen-depleting agent Oxirase (Oxyrase Company, Mansfield, OH) before image acquisition. Tips of individual MTs were manually tracked using MetaMorph software. Parameters of MT dynamic instability were determined by decomposing the trajectories of MT ends into phases of growth, shortening, and pauses using multiscale trend analysis (Lomakin et al., 2009). The length of CLIP-170 comets and the amount of CLIP-170 bound to MT ends were measured as described previously (Lomakin et al., 2011). For quantification of expression levels of XMAP4 mutants, images of 100 cells expressing GFP-tagged phosphomimetic or nonphosphorylatable mutant were acquire using a fluorescein filter set, and the values for average gray levels within the cell outlines were determined using the Region Measurements Tool of MetaMorph software. For quantification of levels of XMAP4 bound to MTs, images of 20 cells stimulated with either melatonin or MSH immunostained with XMAP4 MBD antibodies and rhodamine-conjugated secondary antibodies were acquired using a rhodamine filter set, and the MetaMorph Region Measurements Tool was used to quantify average fluorescence over five ~1-μm MT segments in each cell. After background subtraction, the results of fluorescence measurements were averaged separately for
Stochastic computational model for pigment aggregation

Computational modeling of pigment aggregation was performed as described previously (Lomakin et al., 2009, 2011).

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health Grants GM62290 (to V.I.R.) and P41RR013186 (to V.I. and A.C.).

REFERENCES


Chem 271, 10834–10843.


Tortora E, Galjart N, Avila J, Sayas CL (2013), MAP1B regulates microtubule dynamics by sequestering EB1-3 in the cytosol of developing neuronal cells. EMBO J 32, 1293–1306.