

Melanophilin and myosin Va track the microtubule plus end on EB1

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In mouse melanocytes, myosin Va is recruited onto the surface of melanosomes by a receptor complex containing Rab27a that is present in the melanosome membrane and melanophilin (Mlp), which links myosin Va to Rab27a. In this study, we show that Mlp is also a microtubule plus end-tracking protein or +TIP. Moreover, myosin Va tracks the plus end in a Mlp-dependent manner. Data showing that overexpression and short inhibitory RNA knockdown of the +TIP EB1 have opposite effects on Mlp-microtubule interaction, that Mlp interacts

directly with EB1, and that deletion from Mlp of a region similar to one in the adenomatous polyposis coli protein involved in EB1 binding blocks Mlp's ability to plus end track argue that Mlp tracks the plus end directly by hitchhiking on EB1. These results identify a novel +TIP and indicate that vertebrate cells possess a +TIP complex that is similar to the Myo2p-Kar9p-Bim1p complex in yeast. We suggest that the +TIP complex identified in this study may serve to focus the transfer of melanosomes from microtubules to actin at the microtubule plus end.

Introduction

Visible pigmentation in mammals requires that melanocytes donate melanosomes, their specialized pigment-producing organelle, to keratinocytes. For this intercellular transfer to be effective, melanosomes must first accumulate at the distal end of the melanocyte's dendritic extensions, which are the sites of transfer. Melanocytes generate this peripheral accumulation of melanosomes using a cooperative transport mechanism in which long-range, bidirectional, microtubule-dependent melanosome movements along the length of dendrites are coupled to myosin Va-dependent capture and local movement of the organelles within distal actin-rich regions of the dendrite (Wu et al., 1998). Myosin Va is recruited onto the melanosome surface by a receptor complex containing Rab27a, which is anchored in the melanosome membrane, and melanophilin (Mlp), which links Rab27a to myosin Va by binding Rab27a in a GTP-dependent fashion through its NH₂ terminus and myosin Va through sequences present in the middle of the protein (Fukuda et al., 2002; Strom et al., 2002; Wu et al., 2002a). The absence of any one of these three proteins collapses the myosin Va-dependent capture of melanosomes in the periphery, causing their accumulation in the central cytoplasm.

Microtubule plus end-tracking proteins or +TIPs appear by time-lapse microscopy to track or "surf" the plus end of growing microtubules (Carvalho et al., 2003). These proteins, which include CLIP-170, EB1, adenomatous polyposis coli protein (APC), dynein, and numerous proteins that interact with dynein, have been implicated in the regulation of microtubule dynamics, the loading of vesicular cargo for dynein-dependent movement, and the orientation of the microtubule-organizing center (MTOC) and mitotic spindle (Gundersen et al., 2004). +TIPs accumulate at the microtubule plus end via a treadmill mechanism, by binding to or "hitchhiking" on another +TIP that is treadmilling, and/or by kinesin-dependent translocation. In this study, we show that Mlp is also a +TIP, that it recruits myosin Va to the plus end as well as to the melanosome, and that it plus end tracks by hitchhiking on EB1. These additional properties may allow Mlp to focus and facilitate the transfer of melanosomes from the microtubule to actin at the microtubule plus end.

Results and discussion

Mlp is a +TIP

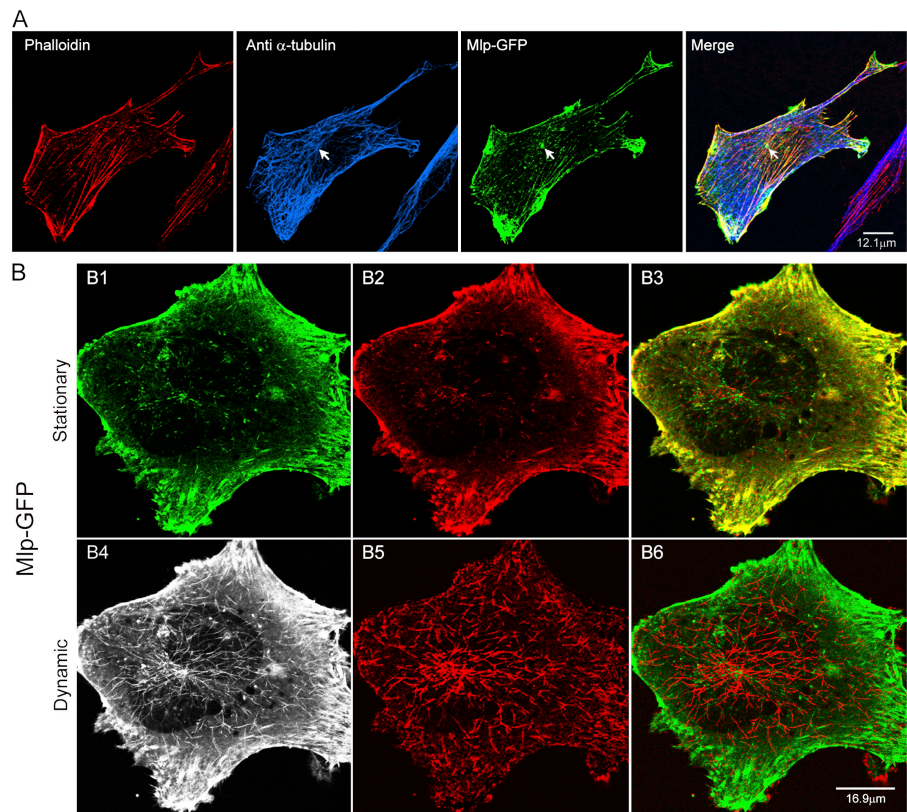
While using full-length GFP-tagged Mlp (Mlp-GFP) to determine the protein's distribution in primary melanocytes, we noticed that, in addition to melanosomes, Mlp showed variable targeting to three apparent cytoskeletal structures: actin stress fibers, cortical actin, and a single perinuclear spot presumably corresponding to the MTOC (unpublished data). These cyto-

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Abbreviations used in this paper: APC, adenomatous polyposis coli protein; MBP, maltose-binding protein; Mlp, melanophilin; MTOC, microtubule-organizing center; RBD, Rab27a-binding domain; siEB1, short inhibitory RNA for EB1.

The online version of this article contains supplemental material.

Figure 1. Cytoskeletal targeting of Mlp. (A) Mlp-GFP targets to actin stress fibers, cortical actin, and the MTOC (arrows) in fixed fibroblasts. (B) B1–B6 are designed to show (using still images) that Mlp-GFP exhibits a relatively stationary signal that is associated with cortical actin and a dynamic signal that is associated with microtubule plus ends [Video 1, available at <http://www.jcb.org/cgi/content/full/jcb.200503028/DC1>]. A fibroblast expressing Mlp-GFP was imaged at 1 frame/s for 30 s. B1 and B2 are the first and last frames (B2 is pseudocolored red), whereas B3 is the merge of these two images. The prominent yellow shows where Mlp has not changed position over 30 s. B4 shows a projection of all 30 frames, whereas B5 (pseudocolored red) shows the signal remaining in the projected image after subtraction of the first frame (B1). B6 shows the merge of B1 and B5. The dynamic microtubule-associated signal for Mlp-GFP appears in red, whereas the stationary actin-associated signal appears in green.



skeletal-like distributions were even more pronounced in primary fibroblasts that contaminated the melanocyte cultures. Staining of transfected fibroblasts with phalloidin and an antibody to α -tubulin confirmed that Mlp-GFP concentrates on actin stress fibers, cortical actin, and at the MTOC (Fig. 1 A). To extend these observations, we examined the dynamic behavior of Mlp-GFP in fibroblasts (Fig. 1 B and Video 1, available at <http://www.jcb.org/cgi/content/full/jcb.200503028/DC1>). Time-lapse images contained two distinct types of fluorescent signal: nearly stationary fluorescence that appeared to correspond to the actin-rich structures (Fig. 1, B1–B3) and, to our surprise, highly dynamic cometlike fluorescent signals radiating from the centrosome (Fig. 1, B4–B6; and Video 1). These latter structures emanated continuously from the bright spot at the MTOC and moved in a persistent, roughly linear path to the cell periphery in a fashion similar to that described previously for +TIPs (Carvalho et al., 2003).

We used four approaches to prove that Mlp is a +TIP. First, we showed that in fibroblasts cotransfected with mRFP-tagged Mlp (Mlp-mRFP) and GFP-tagged α -tubulin, Mlp comets localized at the distal end of microtubules and remained there as the microtubules grew (Fig. 2 A and Video 2, available at <http://www.jcb.org/cgi/content/full/jcb.200503028/DC1>). Second, we showed that in fibroblasts cotransfected with Mlp-mRFP and EB1-GFP, which is a well-characterized +TIP, the two proteins tracked together throughout the cell (Fig. 2 B and Video 3, available at <http://www.jcb.org/cgi/content/full/jcb.200503028/DC1>). As reported previously (Mimori-Kiyosue et al., 2000), we observed that EB1-GFP comets disappear when growing microtubules reach the edge of the cell because

EB1 does not remain at the end of pausing or shrinking microtubules. In most cases (Fig. 1 B and Video 1), we could not be certain that Mlp-GFP also disappeared at the periphery because the abundant signal from Mlp-GFP that is associated with cortical actin usually obscured the protein's microtubule plus end signal near the cell margin. Where this was not a problem (e.g., the cell in Video 3), Mlp and EB1 blinked out together, suggesting that Mlp, like EB1, does not usually associate with the plus ends of pausing or shrinking microtubules. Third, we found that Mlp-GFP comets moved at a uniform rate of $0.23 \pm 0.1 \mu\text{m/s}$ ($n = 100$ from five cells), which is very similar to the rate of microtubule growth reported previously using the +TIPs CLIP-170 (Komarova et al., 2002) and EB1 (Mimori-Kiyosue et al., 2000) as reporters. Fourth, we showed that a low dose of nocodazole (100 nM), which leaves the interphase microtubule array largely intact but blocks growth at the plus end and dissociates +TIPs, caused Mlp-GFP comets to vanish within 1 min (Fig. 2 C and Video 4).

We found that Mlp-GFP exhibited robust plus end tracking behavior in other cell types, including normal rat kidney fibroblasts, COS, CV1, and HeLa (unpublished data). That said, none of these cell types possess detectable levels of endogenous Mlp despite the fact that Mlp mRNA is present in a wide range of mouse tissues (Matesic et al., 2001). In contrast, Mlp is highly expressed in melanocytes. Given this and the fact that Mlp's role as an adaptor protein for organelle–myosin Va interaction was established in melanocytes, we sought to characterize the dynamic behavior of Mlp in these cells. Fig. 3 A and Video 5 (available at <http://www.jcb.org/cgi/content/full/jcb.200503028/DC1>) show that Mlp-GFP exhibits clear plus

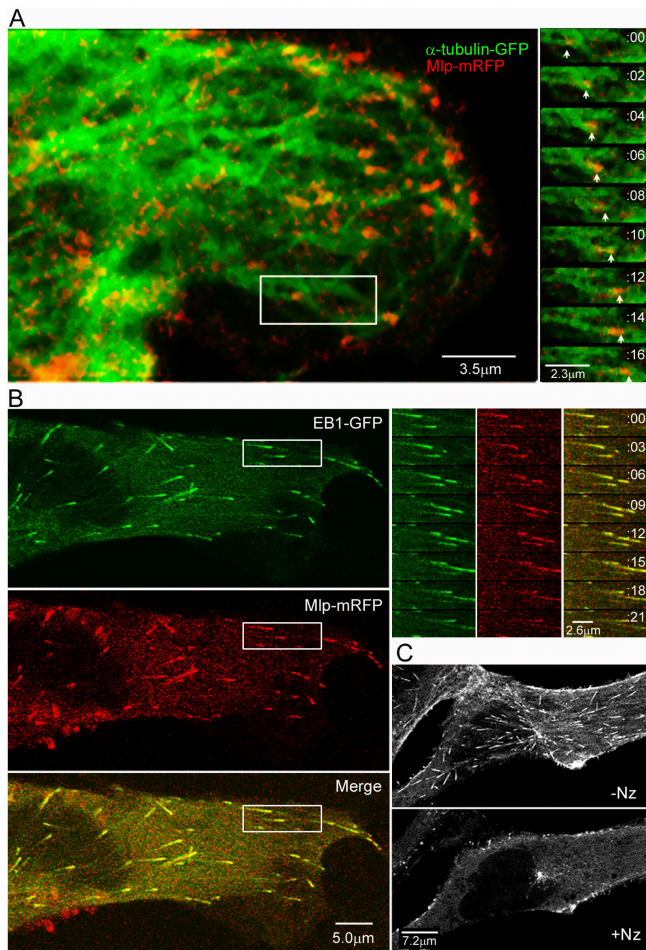


Figure 2. Mlp is a +TIP. (A) Mlp remains at the end of growing microtubules (Video 2, available at <http://www.jcb.org/cgi/content/full/jcb.200503028/DC1>). A time series inside the boxed region is shown to the right (arrows mark the growing microtubule end). (B) Mlp and EB1 plus end track together (Video 3). A time series of the boxed regions is shown to the right. (C) Mlp comets disappear within 1 min after the addition of 100 nM nocodazole (Nz; Video 4).

end tracking behavior in primary wild-type melanocytes in addition to targeting to melanosomes. Mlp-GFP also tracked the plus end in a variety of melanocyte cell lines, including melan-c melanocytes that make unpigmented melanosomes (see below). Although transfected melanocytes overexpressed Mlp-GFP an average of ~ 12 -fold based on Western blotting (Fig. S1 A, available at <http://www.jcb.org/cgi/content/full/jcb.200503028/DC1>), correlative time-lapse microscopy coupled with quantitative immunofluorescence staining using Mlp antibody to detect both endogenous Mlp and overexpressed Mlp-GFP showed that individual transfected melanocytes can show prominent Mlp plus end tracking behavior with less than twofold overexpression (i.e., without huge overexpression; Fig. S2 A and Video 6). Although 67% ($n = 300$) of transfected melanocytes showed targeting of Mlp-GFP to both melanosomes and the plus end, the remaining cells showed almost exclusive targeting to either the plus end/actin or to melanosomes (Fig. 3, B and C; and Video 7). We do not know the basis for this differential targeting. Finally, melanosomes do not plus end track.

Whether visualized with transmitted light or as Mlp-GFP-tagged structures (Fig. 3, A and C; Wu et al. 1998, 2002a), the properties of their movement (intermittent, bidirectional, and ~ 1 – $1.5 \mu\text{m/s}$) are quite distinct from those of +TIPs (persistent, unidirectional, and $\sim 0.25 \mu\text{m/s}$).

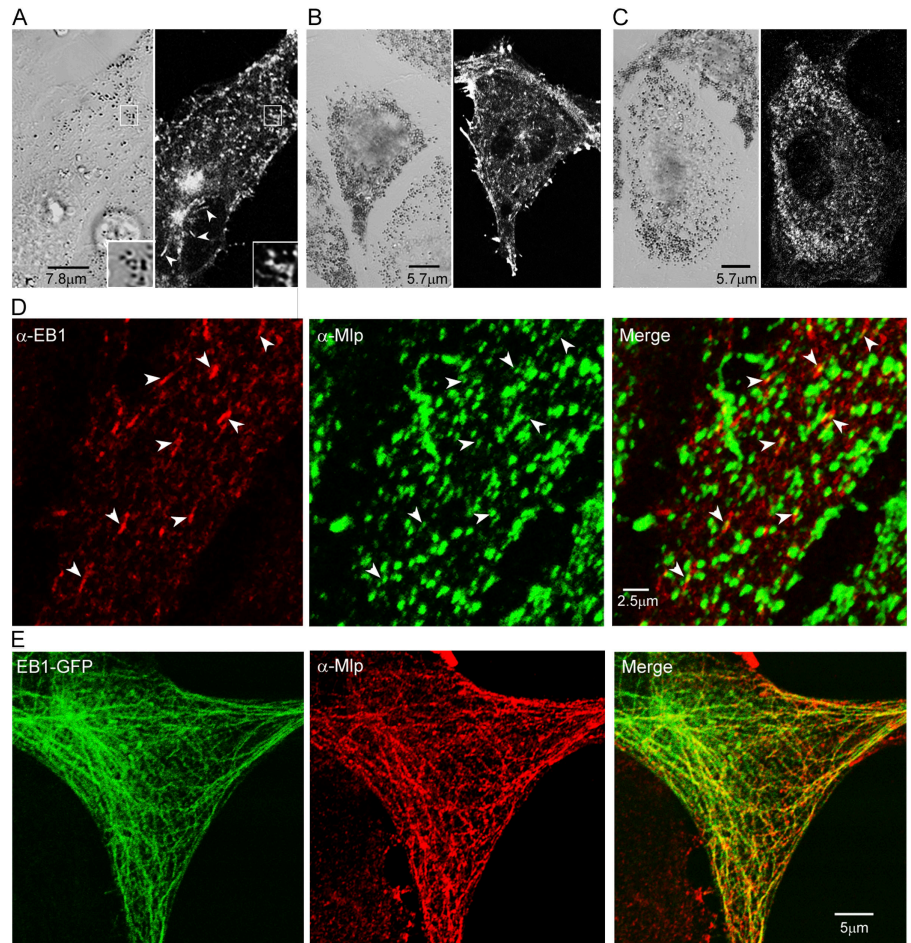
Two experiments showed that Mlp does not need to interact with Rab27a in order to plus end track. First, a version of Mlp containing a single amino acid change (E15A) that abrogates its interaction with Rab27a (Mlp(-) Rab27a-binding domain [RBD]-GFP; Kuroda et al., 2003) showed dramatic plus end tracking behavior in wild-type melanocytes (Fig. S2 B). Second, Mlp-GFP exhibited normal plus end tracking behavior in ashen melanocytes (Fig. S2 B), which are devoid of Rab27a. Mlp also does not need to interact with myosin Va in order to plus end track because Mlp-GFP tracked normally in dilute melanocytes (Fig. S2 C), which are devoid of myosin Va, and Mlp(-) myosin Va-binding domain (MBD)-GFP, which is a version of Mlp containing four closely spaced amino acid changes (D378A, E380A, E381A, and E382A) that abrogate its interaction with myosin Va (Kuroda et al., 2003), tracked normally in wild-type melanocytes (Fig. S2 C).

Double staining of melan-c melanocytes with antibodies to EB1 and Mlp showed that a subset of EB1 comets contain Mlp staining (Fig. 3 D). Of 595 EB1 comets in nine cells, $22.0 \pm 7.6\%$ contained Mlp staining. As a control for random overlap between EB1 comets and Mlp-positive melanosomes, cells were double stained for EB1 and Rab27a because Rab27a is required for the targeting of Mlp to melanosomes (Wu et al., 2002a) and because Rab27a itself does not surf (see below). $7.0 \pm 3.0\%$ of 494 EB1 comets in 10 cells contained Rab27a staining ($P < 0.00002$ vs. $22.0 \pm 7.6\%$). We conclude, therefore, that a small subset ($\sim 15\%$) of endogenous EB1 comets contain endogenous Mlp. We also found that in cells overexpressing EB1-GFP, in which EB1 decorates the entire microtubule lattice, endogenous Mlp can be recruited along the length of the microtubule (Fig. 3 E and see Fig. 5).

Myosin Va tracks the microtubule plus end in a Mlp-dependent manner

Although myosin Va and Rab27a are not required for Mlp to plus end track, one or both proteins might still track together with Mlp. To address this question, we used CV1 cells because they do not express Mlp, thereby allowing us to address the Mlp dependency of any possible plus end tracking behavior exhibited by myosin Va or Rab27a. When we cotransfected CV1 cells with Mlp-mRFP and a GFP-tagged version of the full-length melanocyte-spliced heavy chain isoform of myosin Va (MCMVa-GFP), which is fully capable of rescuing dilute melanocytes (Wu et al., 2002b), we observed a striking colocalization of the two proteins on comets whose dynamics were largely indistinguishable from those of Mlp alone (Fig. 4 A and Video 8, available at <http://www.jcb.org/cgi/content/full/jcb.200503028/DC1>). Moreover, both the myosin Va and Mlp components of these comets disappeared within 1 min after the addition of 100 nM nocodazole (not depicted). Importantly, CV1 cells that were transfected with MCMVa-GFP alone (Fig. S2 D) never exhibited GFP-labeled comets ($n = 60$

Figure 3. Mlp plus end tracks in melanocytes. (A) In a typical transfected melanocyte, Mlp-GFP targets to both melanosomes (inset) and microtubule plus ends (arrowheads; Video 5, available at <http://www.jcb.org/cgi/content/full/jcb.200503028/DC1>). (B and C) Examples in which Mlp-GFP targets almost exclusively to microtubule plus ends/actin (B) or to melanosomes (C; Video 7). (D) A subset of endogenous EB1 comets stain for endogenous Mlp (arrowheads), whereas endogenous Mlp is recruited along the length of microtubules in melanocytes overexpressing EB1-GFP (E).



cells in five independent experiments). Together, these data demonstrate that myosin Va can track the plus end of growing microtubules and argue that this behavior is strictly Mlp dependent. In contrast, Rab27a-GFP, which is fully capable of rescuing ashen melanocytes (Wu et al., 2002a), did not exhibit plus end tracking behavior in CV1 cells when coexpressed with Mlp-mRFP ($n = 45$ cells in three independent experiments; unpublished data), indicating that Rab27a does not track together with Mlp.

The ability of myosin Va to interact with Mlp that is present on melanosomes has been shown to require exon F, one of two alternatively spliced exons that are inserted into the central stalk domain of the melanocyte-spliced heavy chain isoform of myosin Va (the other being exon D; Wu et al., 2002a,b). The ability of myosin Va to plus end track with Mlp also appears to be exon F dependent, as the melanocyte-spliced isoform lacking exon D (MCMVa(-)D-GFP) plus end tracks in CV1 cells that are cotransfected with Mlp-mRFP, whereas the melanocyte-spliced isoform lacking exon F (MCMVa(-)F-GFP) as well as the brain-spliced isoform (BRMVa-GFP), which lacks both exons D and F, do not ($n = 40$ cells each in two independent experiments; Fig. S2 D). Consistent with these results, the tail domain of the melanocyte-spliced isoform was sufficient to plus end track in a Mlp-dependent manner (Fig. 4 B).

Mlp tracks the plus end indirectly by hitchhiking on EB1

The yeast class V myosin Myo2p associates with the microtubule plus end by binding to Kar9p, which, in turn, binds to Bim1p, the yeast homologue of EB1 (Yin et al., 2000). Although Mlp and Kar9p are not considered to be homologues, the results in yeast led us to examine whether plus end tracking by Mlp and, by extension, myosin Va is also EB1 dependent. We used four approaches to address this question. First, we examined Mlp-mRFP distribution in cells expressing very high levels of EB1-GFP (Fig. 5, A–C). As reported previously in other cell types (Mimori-Kiyosue et al., 2000), when EB1-GFP was heavily overexpressed in melan-c melanocytes, it localized along the entire length of microtubules instead of just at their plus ends (Fig. 5 B). In these instances, cotransfected Mlp-mRFP also localized along the entire microtubule lattice and showed greatly diminished localization on cortical actin (Fig. 5, A and C). In contrast, melan-c cells expressing high levels of Mlp-GFP alone (i.e., in the presence of endogenous EB1) never showed labeling of the microtubule lattice, and the excess Mlp appeared to target primarily to cortical actin (Fig. 5, D–F). This dramatic increase in the degree of Mlp–microtubule interaction that was caused by the overexpression of EB1, which was also seen for endogenous Mlp in cells overexpressing EB1-GFP (Fig. 3 E),

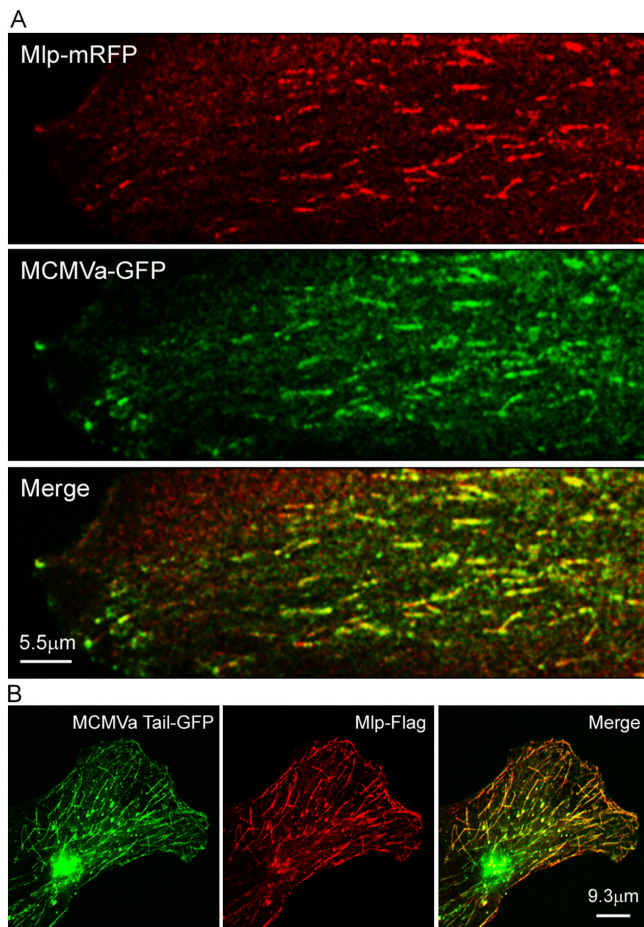


Figure 4. Myosin Va tracks the microtubule plus end. (A) A GFP-tagged version of the melanocyte-spliced isoform of myosin Va (MCMVa-GFP) plus end tracks together with Mlp-mRFP throughout the cell (Video 8, available at <http://www.jcb.org/cgi/content/full/jcb.200503028/DC1>). (B) In a fixed cell, the tail domain of this isoform (MCMVaTail-GFP) targets to microtubule plus ends together with FLAG-tagged Mlp (Mlp-FLAG; visualized by staining with α -FLAG antibody). The omission of Mlp-FLAG abrogated the interaction of MCMVaTail-GFP with microtubules (not depicted).

suggests that normal levels of EB1 might recruit Mlp to microtubule plus ends.

Second, we used RNA interference to reduce the level of endogenous EB1 in melan-c melanocytes (which make EB1 but not EB2 or EB3; Fig. 5 G) and asked whether this compromised the plus end targeting of Mlp. Quantitative Western blotting revealed a reduction in EB1 protein levels of 92% (average of two experiments) in cells that were treated with short inhibitory RNA for EB1 (siEB1) for 48 h relative to mock-treated cells (Fig. 5 H). Consistent with this, the vast majority of siEB1-treated cells did not contain obvious EB1-positive comets when stained for endogenous EB1 at 48 h (unpublished data). In parallel 48-h cultures, we then introduced GFP-tagged Mlp, waited 24 h, and scored for the frequency of Mlp plus end tracking by time-lapse microscopy. Mlp(-)RBD-GFP was used to maximize the likelihood of observing this behavior. Unequivocal Mlp plus end tracking was detected in only 22% of transfected, siEB1-treated cells as compared with 90% of transfected, mock-treated cells ($n =$

50 cells each in an average of two experiments; Video 9, available at <http://www.jcb.org/cgi/content/full/jcb.200503028/DC1>). In an effort to eliminate the possibility that this difference was caused simply by a large reduction in the number of growing microtubule ends in EB1 knockdown cells, we transfected both siEB1- and mock-treated cells with GFP-tagged CLIP-170, which can track the plus end without EB proteins (Carvalho et al., 2003). Although the appearance and dynamics of GFP-CLIP-170 was altered by EB1 knockdown (more diffuse signal and less robust comets), unequivocal GFP-CLIP-170 plus end tracking behavior was still found in 86% of siEB1-treated cells versus 90% of mock-treated cells ($n = 50$ cells each in an average of two experiments; Video 9). We conclude, therefore, that the absence of Mlp(-)RBD-GFP comets in the majority of siEB1-treated melanocytes primarily reflects an EB1 dependency for Mlp plus end tracking rather than a dramatic decrease in the frequency of growing microtubule ends.

The data discussed above argue that there might be a physical interaction between EB1 and Mlp. Therefore, as a third test, we asked whether beads coated with EB1 fused to GST bind Mlp that is present in lysates of SF9 cells expressing full-length FLAG-tagged Mlp (Mlp-FLAG). Fig. 5 I shows that GST-EB1 beads but not GST beads bound an 88-kD protein that was visible in Coomassie blue-stained samples and corresponded to the molecular weight of Mlp-FLAG. Moreover, the amount of this 88-kD protein increased as increasing amounts of EB1-GST beads were added to binding reactions containing a constant amount of SF9 cell lysate. Proof that the 88-kD band is Mlp-FLAG and that GST-coated beads do not bind any Mlp-FLAG was obtained by probing the same samples with antibodies to Mlp and the FLAG tag (Fig. 5 I, top). To demonstrate that this apparent interaction between EB1 and Mlp is direct, pull-down assays were repeated using Mlp-FLAG that was first purified to homogeneity from SF9 cells (Fig. 5 K). By both Coomassie blue staining (Fig. 5 J) and Western blot analyses (Fig. 5 J, top), EB1-GST beads but not GST beads bound pure Mlp-FLAG, indicating that Mlp interacts directly with EB1. These results argue that Mlp functions like Kar9p to link a type V myosin to EB1.

Given the results described above, as a fourth test, we examined the sequence of Mlp for the presence of the region shared between Kar9 and APC that is involved in their interaction with EB proteins (Slep et al., 2005). Fig. 5 L shows that the COOH-terminal 100 residues of Mlp (residues 491–590), which follow a short, predicted coiled coil (schematic), can be aligned with a portion of the COOH-terminal region of APC that is implicated in its binding to EB1 (see Fig. 5 for details). We deleted this entire region from Mlp, creating Mlp1-490-GFP. CV1 cells expressing this fusion, which stably accumulates in cells (Fig. S1 B), never exhibited GFP-labeled comets ($n = 60$ cells in three independent experiments; Video 10, available at <http://www.jcb.org/cgi/content/full/jcb.200503028/DC1>). In contrast, CV1 cells expressing a fusion of GFP to Mlp residues 491–590 (Mlp491–590-GFP) exhibited robust GFP-positive comets (Video 10). Consistent with these results, pull-down experiments from doubly trans-

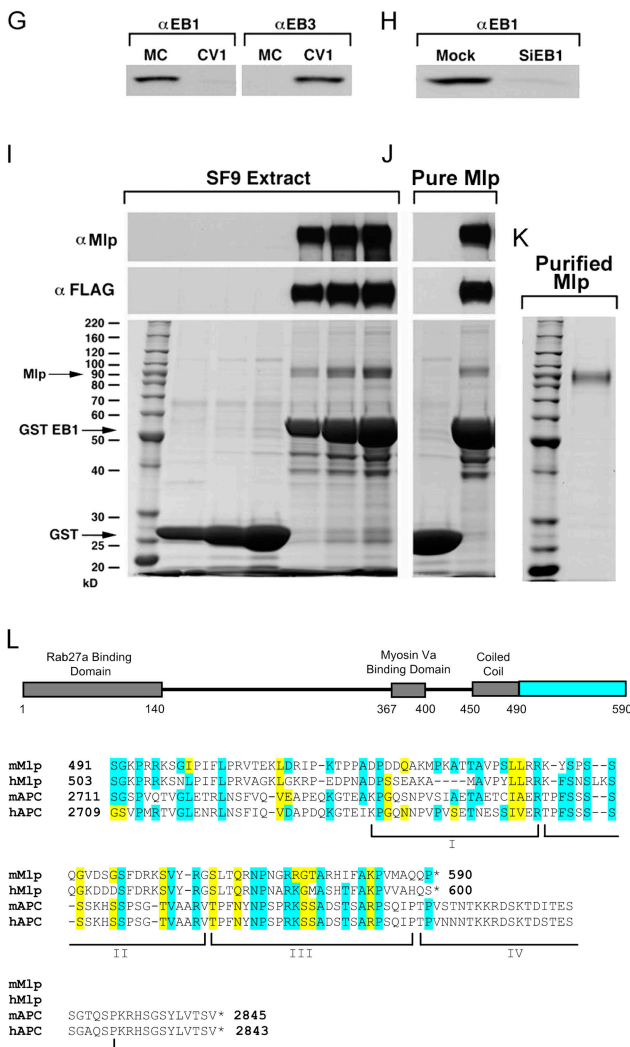
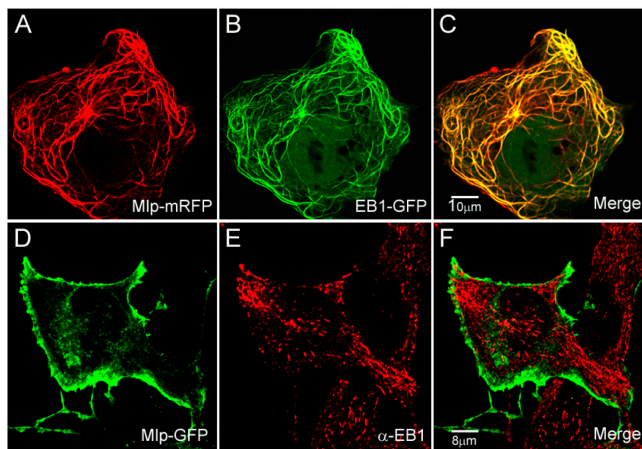


Figure 5. Mlp hitchhikes on EB1. (A–F) The overexpression of EB1-GFP recruits Mlp-mRFP onto the length of microtubules (A–C), but this is not seen when Mlp-GFP is overexpressed by itself (D–F; Fig. S2 E shows that Mlp-GFP is not recruited nonspecifically to taxol-stabilized microtubules; available at <http://www.jcb.org/cgi/content/full/jcb.200503028/DC1>). (G) Melanocytes make EB1 but not EB3 (CV1 cells do the converse). Blots probed with antibody to EB2/RP1 failed to detect this protein in melanocyte (MC) extracts (not depicted). (H) The reduction in cellular EB1 level after short inhibitory RNA treatment. (I and J) GST-EB1 but not GST binds Mlp-FLAG whether present in SF9 whole cell extracts (I) or as a pure protein

fectured COS cells showed that full-length FLAG-tagged Mlp, but not Mlp residues 1–490 tagged with FLAG, interacts with EB1-GFP in vivo (Fig. S1 C). Moreover, the COOH-terminal 100 residues of Mlp that are expressed as a maltose-binding protein (MBP) fusion interact with EB1-GST in vitro (Fig. S1 D). Together, these results identify within Mlp a sequence that is implicated in APC–EB1 interaction, show that the deletion of this sequence blocks Mlp’s ability to plus end track and interact with EB1, and demonstrate that this sequence, by itself, acts as a +TIP and binds EB1.

All of our data argue that Mlp and, by extension, myosin Va track the microtubule plus end indirectly by hitchhiking on EB1, whose accumulation at the growing end is probably mediated by treadmilling (Carvalho et al., 2003). Moreover, Mlp appears to have no ability on its own to bind to microtubules, which is in contrast to other +TIPs. We also never observed “backtracking” of Mlp comets, which occurs for proteins whose plus end accumulation is a result, at least in part, of delivery by kinesins (Carvalho et al., 2004). Nevertheless, mechanisms other than hitchhiking on EB1 might still make some contribution to Mlp’s plus end accumulation.

Only a subset of EB1 comets stained for endogenous Mlp. We think this is primarily caused by the extensive targeting of endogenous Mlp to melanosomes, which limits the pool of free Mlp that is available for treadmilling with EB1. Factors that influence the Rab27a-dependent recruitment of Mlp to melanosomes, such as the guanine nucleotide exchange factor and GTPase-activating protein for Rab27a, may dramatically influence the plus end targeting of endogenous Mlp. Other factors that may affect Mlp’s plus end targeting include competition with other +TIPs for binding to EB1, phosphorylation, and, as suggested for Kar9 (Kusch et al., 2003), the self-association of Mlp. Exactly how the partitioning of Mlp between melanosomes, microtubule plus ends, and actin is regulated and interconnected remains to be determined.

At present, we do not know what aspects of Mlp’s overall function are specifically dependent on its ability to plus end track. Given Mlp’s role in coupling Rab27a-positive melanosomes to myosin Va, we suggest that a plus end complex of EB1–Mlp–myosin Va might serve to focus and, in some way, mechanistically facilitate the transfer of melanosomes from microtubules to actin at this location (Fig. S3, available at <http://www.jcb.org/cgi/content/full/jcb.200503028/DC1>). Focusing track switching at this site (i.e., at dendritic tips where melanosome transfer to keratinocytes occurs) would further drive mammalian pigmentation.

(J; bottom, Coomassie blue; top, Westerns with α -FLAG or α -Mlp antibodies). (K) Purified Mlp. (L) Schematic of Mlp and an alignment of COOH-terminal sequences in Mlp and APC (blue region; m, mouse; h, human). Only residues that are shared between Mlp and APC are highlighted (blue, identity; yellow, conservative substitution). Brackets (I–IV) show the positions of four pseudorepeats in APC (Slep et al., 2005). Mlp and APC have no similarity outside of the region aligned in L, and Mlp lacks APC repeat IV, which is critical for APC–EB1 interaction. Asterisks indicate COOH-terminal ends. Numbers below the schematic indicate the residue number in Mlp. Numbers in bold (bottom) indicate the beginning and ending residue numbers.

Materials and methods

Cell culture and microscopy

Primary mouse melanocytes and fibroblasts as well as melan-c melanocytes (gift of D. Bennet, St. George's Hospital Medical School, London, UK), CV1, COS, HeLa, and normal rat kidney fibroblast cells were cultured and transfected as described previously (Wu et al., 2002a,b). Rhodamine-phalloidin and the antibody to α -tubulin were purchased from Invitrogen. Antibodies to EB1 and EB3 were purchased from Transduction Labs. The antibody to EB2/RP1 was provided by C. Renner (Saarland University Medical School, Homburg, Germany). The α -Mlp and α -Rab27a antibodies were prepared previously (Wu et al., 2002a). Nocodazole was purchased from Sigma-Aldrich. Cells were fixed and stained as described previously (Wu et al., 2002b) except that they were immersed in -20°C methanol for 5 min before fixation with PFA. For colocalization of endogenous EB1 and Mlp, cells were rapidly frozen in liquid propane and slowly freeze substituted into methanol/formaldehyde as described previously (Rochlin et al., 1995). Confocal microscopy was performed on a microscope (LSM 510; Carl Zeiss Microimaging, Inc.) as described previously (Wu et al., 2002a).

Constructs

Mlp-GFP, Mlp-FLAG, MCMVa-GFP, various spliced versions of GFP-tagged myosin Va, MCMVaTail-GFP, and Rab27a-GFP were described previously (Wu et al., 2002a). EB1-GFP, GFP-CLIP-170, and GFP- α -tubulin were gifts from H. Goodson (University of Notre Dame, Notre Dame, Indiana), Y. Kiyosue (Knowledge Action Network Research Institute, Kyoto, Japan), and G. Patterson (National Institute of Child Health and Human Development, Bethesda, MD), respectively. Transfers of genes to different color vectors (BD Biosciences) as well as the construction of GST-EB1, FLAG-tagged Mlp in the baculoviral vector pVL1392, Mlp1-490-GFP or FLAG, Mlp491-590-GFP, and Mlp491-590-MBP were performed by standard techniques. Mlp(-)RBD-GFP and Mlp(-)MBD-GFP were constructed as described previously (Kuroda et al., 2003).

Short inhibitory RNA and pull-down experiments

Cells were transfected with the siEB1 oligonucleotide (5'-AUGUGUACUC-UACGUCAGU-3'; final concentration of 100 nM) using LipofectAMINE 2000 (Invitrogen). Quantitative Western blotting, the expression and purification of FLAG-tagged Mlp from SF9 cells, GST and MBP pull-down experiments, and COS cell pull downs with anti-FLAG M2 beads were performed as described previously (Wu et al., 2002a,b).

Online supplemental material

Fig. S1 shows the estimation of Mlp over expression and the interaction of Mlp and Mlp fragments with EB1 in vitro and in vivo. Fig. S2 shows the correlation between Mlp plus end tracking behavior and overexpression and the plus end tracking behavior of Mlp mutants and various spliced isoforms of myosin Va. Fig. S3 shows a model for how the plus end complex of EB1-Mlp-myosin Va might serve to focus the transfer of melanosomes from microtubules to actin at the microtubule plus end. Video 1 shows the dynamic behavior of Mlp-GFP in a primary mouse fibroblast. Video 2 shows that Mlp-mRFP tracks the growing end of microtubules that are visualized with GFP-tubulin. Video 3 shows that EB1-GFP and Mlp-mRFP track the plus end together. Video 4 shows that a low dose of nocodazole blocks Mlp-GFP plus end tracking. Video 5 shows Mlp-GFP in a primary wild-type mouse melanocyte where the protein has targeted to both the melanosome and the microtubule plus end. Video 6 shows Mlp-GFP plus end tracking behavior in a lightly transfected melan-c melanocyte that was used for correlative video/quantitative immunofluorescence. Video 7 shows Mlp-GFP in primary wild-type melanocytes where the protein has targeted almost exclusively to either the plus end/actin or to melanosomes. Video 8 shows that Mlp-mRFP and MCMVa-GFP track the plus end together. Video 9 shows the dynamics of Mlp(-)RBD-GFP and GFP-CLIP-170 in melan-c melanocytes that were either mock or siEB1 treated. Video 10 shows that Mlp491-590-GFP tracks the plus end, whereas Mlp1-490-GFP does not. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200503028/DC1>.

We thank Takehito Uruno for identifying the APC-like sequence in Mlp, Gregg Gundersen and David Pellman for advice, and Kevin Slep and Ron Vale for providing information before publication.

Submitted: 7 March 2005

Accepted: 15 September 2005

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