Microtubule plus-end loading of p150\textsuperscript{Glued} is mediated by EB1 and CLIP-170 but is not required for intracellular membrane traffic in mammalian cells

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Summary
Microtubule dynamics and function are regulated, at least in part, by a family of proteins that localize to microtubule plus-ends, and include EB1, CLIP-170 and the dynactin component p150\textsuperscript{Glued}. Plus-end pools of these proteins, notably dynactin, have been invoked in a number of ‘search-and-capture’ mechanisms, including the attachment of microtubules to kinetochores during mitosis and to endomembranes prior to the initiation of intracellular transport. Here we show that, in mammalian cells, EB1 is required for the plus-end localization of CLIP-170, and that this is in turn required to localize p150\textsuperscript{Glued} to plus-ends. Specific depletion of CLIP-170 results in defects in microtubule dynamics, cell polarization in response to scratch wounding and a loss of p150\textsuperscript{Glued} from plus-ends. By contrast, removal of p150\textsuperscript{Glued} from plus-ends by depletion of either EB1 or CLIP-170 caused no defects in the localization of intracellular organelles, the dynamics of ER-to-Golgi transport, the efficiency of transferrin uptake or the motility of early endosomes or lysosomes. In addition to labelling microtubule plus-ends, we show that GFP-p150\textsuperscript{Glued} becomes incorporated into the dynactin complex and labels small, highly dynamic, punctate structures that move along microtubules. A subset of these structures colocalizes with ER-Golgi transport intermediates. Together, these data show that the function of CLIP-170 and p150\textsuperscript{Glued} in membrane trafficking is not associated with their plus-end localization.

Key words: Microtubule, Dynactin, EB1, CLIP-170, +TIP

Introduction
Dynactin is a large multi-protein complex that interacts with dynein and has been implicated in multiple membrane trafficking steps (Burkhardt et al., 1997; Gill et al., 1991; Presley et al., 1997; Watson et al., 2005). A core component of dynactin, p150\textsuperscript{Glued}, is a microtubule (MT) plus-end tracking protein (+TIP) involved in multiple membrane trafficking steps (Burkhardt et al., 1997; Habermann et al., 2001; Valetti et al., 1999; Vaughan et al., 2002); another +TIP, CLIP-170, has itself been implicated in the motility of early endosomes (Pierre et al., 1992). We have recently shown that p150\textsuperscript{Glued} can interact directly with the COPII complex and that this interaction underlies functional coupling of ER export to the MT cytoskeleton (Watson et al., 2005). Timelapse imaging revealed that MT plus-ends labelled with p150\textsuperscript{Glued} appear to target directly towards ER exit sites (Watson et al., 2005). In line with other ‘search-and-capture’ mechanisms that have been proposed (Mimori-Kiyosue and Tsukita, 2003; Vaughan, 2005), these and other data (Vaughan et al., 2002) suggest that it is this plus-end pool of dynactin that is involved in ER-to-Golgi membrane traffic (Vaughan, 2005). Dynactin and another +TIP, EB1, interact directly (Askham et al., 2002); CLIP-170 also interacts both with EB1 and dynactin (Goodson et al., 2003). The fission yeast homologues of EB1 (mal3p) and CLIP-170 (tip1p) also interact in vitro, and mal3p is required to load tip1p onto MT plus-ends (Busch and Brunner, 2004). CLIP-170 is required for the localization of p150\textsuperscript{Glued} at MT plus-ends in mammalian cells (Lansbergen et al., 2004), and EB1 was recently shown to interact with the D\textit{rosophila} CLIP-170 homologue D-CLIP-190 and to load it onto plus-ends (Dzhindzhev et al., 2005). Thus, one can propose a sequential model of loading and stabilization, resulting in plus-end localization of p150\textsuperscript{Glued}.

We set out to determine whether EB1 and/or CLIP-170 are involved in the plus-end localization of p150\textsuperscript{Glued} in mammalian cells, and to define whether ‘search and capture’ with plus-end pools of dynactin plays a role in intracellular membrane traffic. Using RNA interference (RNAi) to deplete mammalian cells of +TIPS, we show that, in HeLa cells, EB1 is required for the localization of CLIP-170 to MT plus-ends and CLIP-170 is required to load p150\textsuperscript{Glued} onto plus-ends. EB1 alone is not sufficient to localize p150\textsuperscript{Glued} to plus-ends in vivo. We go on to show that, despite having significant effects on cell polarization in response to scratch wounding, depletion of either EB1 or CLIP-170 has no effect on a variety of membrane trafficking events. We have also generated a GFP-fusion protein with human p150\textsuperscript{Glued} (GFP-Hsp150\textsuperscript{Glued}) and show that it can incorporate into the dynactin complex. Using this, we identify a population of GFP-p150\textsuperscript{Glued}-positive endomembranes that translocate along MTs and colocalize with ER-to-Golgi transport intermediates. Together, these data
show that plus-end loading of p150Glued is not required for diverse intracellular trafficking events.

**Results**

We have used small interfering RNA (siRNA) depletion by transfection of HeLa cells with siRNA duplexes targeting lamin A/C (as a control), CLIP-170, p150Glued or EB1 followed by immunoblotting with specific antibodies to show effective depletion of each protein by its own duplex (Fig. 1A). Depletion of +TIPs was confirmed with immunofluorescence. p150Glued was depleted to a maximum of 50% but we were able to reduce expression of EB1 and CLIP-170 to >80%, which probably reflects a hypomorphic state. Notably, cells transfected with siRNA targeting p150Glued failed to divide as quickly as control cells, consistent with the known essential function of dynactin in mitosis (Gill et al., 1991). All gels in Fig. 1 are normalized to protein concentration and thus account for this different cell number. We found that one siRNA duplex, targeting CLIP-170 that has been described previously (Lansbergen et al., 2004) (CLIP-170b), also resulted in a significant depletion of EB1. This was not seen with the other siRNA duplexes (CLIP-170a, CLIP-170c, CLIP-170d, see Fig. 1B). This 'off-target' depletion of EB1 was unpredictable from sequence alignment and does not significantly affect the conclusions of the previous study (Lansbergen et al., 2004). However, it does serve to emphasize the importance of using multiple, independent siRNA duplexes in RNAi experiments. In all subsequent experiments CLIP-170a and CLIP-170d siRNA duplexes were used. Transfection with siRNA duplexes using the modified calcium phosphate transfection method (Chen and Okayama, 1988) resulted in 98.8% (n=335 cells) of cells being transfected with siRNA, as judged by uptake of fluorescent duplexes targeting lamin A/C (Fig. 1C). Fluorescent lamin-A/C-specific duplexes were effective in depletion (not shown). Immunofluorescence labelling of cells depleted of EB1 with anti-EB1 antibodies (Fig. 1D) and similar experiments with other targets (data not shown), confirmed the efficiency of siRNA transfection and target silencing [98.0% of cells were found to be depleted of EB1 by using this approach (n=200 cells)]. Thus, we can be confident that we identified cells that were depleted in subsequent imaging experiments. Multiple cells were analysed in each experiment as indicated.

Depletion of CLIP-170 results in a loss of dynactin from MT plus-ends (Fig. 2A, enlarged in 2B) (see also Lansbergen et al., 2004). We also found that specific RNAi-mediated depletion of EB1 caused loss of p150Glued from MT plus-ends (Fig. 2A, enlarged in 2B). EB1 has been shown to target to plus-ends independently of dynactin (Goodson et al., 2003) and EB1 can interact directly with p150Glued (Berrueta et al., 1999); therefore EB1 could act independently of CLIP-170 in specifying the localization p150Glued to plus-ends (Hayashi et al., 2005). However, our data suggest that EB1 alone is not sufficient to localize p150Glued to plus-ends because depletion of CLIP-170 does not remove EB1 from plus-ends (Fig. 2C), but removes p150Glued (Fig. 2A,B). Depletion of EB1 resulted in a decreased CLIP-170 localization at plus-ends (Fig. 2D) but variability in our ability to detect CLIP-170 at MT plus-ends
with this antibody meant that quantification was impossible. It remains possible that there is a requirement for both CLIP-170 and EB1 in determining the plus-end localization of p150Glued. Quantitative immunofluorescence of siRNA-transfected cells showed that depletion of either CLIP-170 or EB1 was effective to remove p150Glued from plus-ends. Fig. 3A shows the way in which we quantified these data; representative images of p150Glued localization in cells depleted of lamin A/C, p150Glued, EB1 or CLIP-170 are shown individually in Fig. 3B and as an RGB merge with tubulin in Fig. 3C. Representative quantification of tubulin and p150Glued fluorescence at plus-ends is shown in Fig. 3D. Quantification of plus-end labelling for each siRNA transfection (at least 150 plus-ends from five cells on three different coverslips generated in two independent experiments; these data are consistent with visual inspection of many hundreds of cells of at least ten independent experiments) showed that, EB1 depletion resulted in a 72% reduction of plus-end p150Glued, whereas depletion of CLIP-170 resulted in a 80% reduction (Fig. 3E). The amount of plus-end labelling was negligible in p150Glued depleted cells. These data clearly show that depletion of either EB1 or CLIP-170 causes a loss of p150Glued from plus-ends.

Considerable evidence implicates both CLIP-170 and EB1 in the polarization of cells towards the edge of scratch wounds (e.g. Fukata et al., 2002; Wen et al., 2004). After wounding of human lung epithelial A549-cell monolayers (in which we also confirmed effective depletion of proteins using the same RNAi methods, data not shown), we found that reorientation of the centrosome and Golgi is significantly inhibited following depletion of p150Glued, CLIP-170 or EB1 but not following depletion of lamin A/C (Fig. 4). Scratch-wounding controls are included in which cells were fixed immediately following wounding; this represents the random orientation of cells within the population (theoretically 33%). These data show that these +TIPs are depleted to a sufficient extent to perturb cellular functions in which they are known to be involved.

This work provided us with the ability to test directly the role of 'search-and-capture' mechanisms involving plus-end p150Glued, EB1 and CLIP-170 in membrane traffic. Previous work has shown that depletion of CLIP-170 does not affect the steady-state localization of the Golgi or transferrin-receptor-positive endosomes (Lansbergen et al., 2004); in that study, only fixed cells were analyzed and a siRNA duplex that we show also depletes EB1 was used. We exploited the siRNA approach described above to remove p150Glued from plus-ends to study the organization and, more importantly, the dynamics of intracellular membranes by immunofluorescence and time-lapse imaging. The organization of the early secretory pathway was largely unaffected by depletion of lamin A/C, EB1, CLIP-170 or p150Glued (Fig. 5A,B). We observed a minor realocalization of ERGIC-53 (the ER-Golgi intermediate compartment) and golgin-97 (trans-Golgi network) to a more peripheral distribution following depletion of p150Glued (asterisks in Fig. 5), consistent with the known functions of dynactin in centripetal transport (Burkhardt et al., 1997; Presley et al., 1997).

No differences were observed in the distribution and speed of movement of transferrin-positive endocytic compartments (Fig. 6A shows coloured tracks overlayed onto the first image of the accompanying time-lapse sequence, see supplementary material Movie 1). The accumulation of transferrin-positive endosomes in the juxtanuclear area varies between cells; analysis of >100 cells in each experiment revealed no differences in the proportion that shows this accumulation (45±11%), see also Lansbergen et al. (Lansbergen et al., 2004). Time-lapse imaging showed that, after 10 minutes of transferrin uptake, 68±15% transferrin-positive structures moved >1 μm during the course of 2 minutes in control (lamin-
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A/C-depleted) cells. This is essentially unchanged in cells depleted of p150\(^{\text{Glued}}\), CLIP-170 or EB1 (72±12%, 63±15%, 66±12% of objects moving, respectively). The average speed of moving structures (1.2 \(\mu\)m s\(^{-1}\)) was not significantly different in all cases, neither was the range of speeds (0.1-2.7 \(\mu\)m s\(^{-1}\), Fig. 6B). A slight qualitative decrease in the speed of transferrin-positive structures in p150\(^{\text{Glued}}\)-depleted cells was seen but this change was not found to be statistically significant.

We also examined the distribution and mobility of acidic organelles by using LysoTracker labelling (Fig. 6C, and supplementary material Movie 2). The number of moving organelles was essentially unchanged between samples; during 1 minute of imaging at two frames per second, an average of 30% (±8%) of structures moved >1 \(\mu\)m in control (Lamin A/C depleted) cells with 25% (±10%), 28% (±6%) and 32% (±11%) of objects moving in p150\(^{\text{Glued}}\), CLIP-170 and EB1 depleted cells, respectively. Over longer time frames (in which we observed significant photobleaching of LysoTracker) >90% of LysoTracker-positive structures moved >1 \(\mu\)m in a 5-minute sequence. Fig. 6C shows the tracks of selected objects overlayed onto the first frame of the accompanying time lapse sequence (supplementary material Movie 2). Manual tracking of objects showed that the speed of these objects during their period of movement as well as the range of speeds is essentially unchanged regardless of +TIP depletion, averaging 0.79 \(\mu\)m s\(^{-1}\) (Fig. 6D). Depletion of p150\(^{\text{Glued}}\) results in a small but consistent decrease in accumulation of LysoTracker-positive structures in the juxtanuclear region (data not shown), consistent with the known role of dynactin in the transport of lysosomes (Burkhardt et al., 1997). In the case of both transferrin and LysoTracker labelling the directionality of movement was also indistinguishable.

'Search-and-capture' has also been

Fig. 3. Quantification of plus-end p150\(^{\text{Glued}}\).
Example quantification of plus-end labelling with anti-p150\(^{\text{Glued}}\). (A) Localization of p150\(^{\text{Glued}}\) at MT plus-ends was determined by immunofluorescence and quantified by analysis of fluorescence intensity of tubulin and p150\(^{\text{Glued}}\) along a line as shown in the enlargement. Box, 4 \(\mu\)m square. (B) Localization of p150\(^{\text{Glued}}\) in siRNA transfected cells as indicated. (C) Overlays of the images from B with the corresponding tubulin localization. All panels 42×35 \(\mu\)m in B and C. (D) Graphical representation of fluorescence intensity at plus-ends, calculated as in (A). (E) Quantification of >150 plus-ends for each siRNA transfection (at least ten plus-ends from five cells from three coverslips taken from two independent experiments). The histogram shows that average (mean) intensity values (arbitrary units) with the individual points showing the scatter of the data (notice the close clustering of data points close to the x-axis in EB1 and CLIP-170-depleted cells).
proposed to operate during ER-to-Golgi transport (Vaughan et al., 2002). We monitored the transport of tsO45-G-YFP (Keller et al., 2001) to determine whether there were any changes in the rate of formation or motility of VTCs. At 39.5°C tsO45-G-YFP is unfolded, retained in the ER and can be released in a COPII-dependent manner (Aridor et al., 1995) following a shift to 32°C. The protein then enters VTCs that translocate to the Golgi in a dynein-dynactin dependent manner (Presley et al., 1997; Scales et al., 1997). In all cases (depletion of lamin A/C, p150Glued, CLIP-170 or EB1), 10 minutes after release from the ER (shift from 39.5°C to 32°C), at least 96% of cells showed punctate VTCs translocating to the Golgi complex and significant accumulation of fluorescence in the Golgi (at least 100 cells were analysed from three independent experiments, see Fig. 7A,B and supplementary material Movies 3 and 4). This 10-minute time point eliminates focus drift caused by temperature changes. There is some variability in the rate of formation of VTCs in any population of cells between experiments; however, in control experiments we cannot detect any difference in the average speed (1.1±0.28 μm s⁻¹) at which these structures were translocating through the cytoplasm.

**Fig. 4.** Depletion of p150Glued, EB1 or CLIP-170 disrupts polarization of cells in response to scratch wounding. A549 cells transfected with siRNAs that target lamin A/C, p150Glued, EB1 or CLIP-170 were grown for 72 hours and subsequently scratched to wound the monolayer. After a further 2-hour incubation, the position of the Golgi and centrosome was determined. (A) Example wounded monolayer (lamin A/C siRNA) in which cells have reoriented towards the wound edge (white line). Also shown are dots showing the calculated cell centroid and the mask used to score reorientation. Area shown, 86×69 μm. (B) Percentage of cells showing orientation towards the wound edge was scored for each siRNA as well as for a mock-transfected control and also a scratch control (in which cells were scratched and immediately fixed). Notice that a ‘random’ orientation, as found in the scratch control, would mean 33% of cells polarized towards the edge. Data from three experiments, >100 cells for each transfection are shown; error bars show the standard error of the mean (s.e.m.); *, significance determined using ANOVA (P<0.05).

**Fig. 5.** Analysis of membrane trafficking in +TIP-depleted cells. Cells were transfected with siRNA duplexes targeting lamin A/C, p150Glued, CLIP170 or EB1. After 72 hours, cells were fixed with methanol and processed for immunofluorescence. (A) Localization of golgin-97 (trans-Golgi network) and Sec24Cp (COPII-coated ER exit sites). (B) Localization of ERGIC-53 (ER-Golgi intermediate compartment) and giantin (Golgi). Bar, 10 μm.
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The distribution of object speeds is shown in Fig. 7C. There is again a very slight reduction in the velocity of objects in cells depleted of p150Glued but this was not statistically significant. Similarly, no differences were observed in the rate of formation, number or motility of ER-to-Golgi transport carriers 5 minutes after brefeldin A washout (data not shown). Subsequent reassembly of the Golgi was indistinguishable in +TIP-depleted samples (not shown).

These data strongly suggest that plus-end pools of CLIP-170 and p150Glued are not required for intracellular membrane organization or movement. The logical corollary to the conclusion that plus-end dynactin is not involved in intracellular transport is that, dynactin must mediate intracellular transport through localization to other cellular structures, notably along the length of microtubules. A key point here is that many antibodies directed against p150Glued, including those used here, selectively label the plus-end pool of the protein. We have constructed a human p150Glued-GFP fusion protein (GFP-Hsp150Glued) and determined its dynamics and localization in living and fixed cells. At high expression level, GFP-Hsp150Glued decorates the entire MT network (data not shown) as described for its orthologues (Quintyne et al., 1999; Vaughan et al., 2002; Waterman-Storer et al., 1995). At low expression level, in addition to the expected labelling of plus-ends (Fig. 8A), a non-plus-end pool of dynactin functioning in membrane traffic was visible. We identified small punctate structures moving in the cytoplasm that were positive for GFP-Hsp150Glued (Fig. 8B,C). Tracking these structures in live cells shows that they are not associated with plus-ends but translocate along MTs (Fig. 8B and supplementary material Movie 5); at very low expression levels these structures can be seen to move along convoluted curvilinear trajectories that are not consistent with movement along a single MT (Fig. 8C and supplementary material Movie 6). A key question is whether expressed GFP-Hsp150Glued is incorporated into the dynactin complex. Using sucrose density gradient centrifugation, we showed that a significant proportion of GFP-Hsp150Glued is incorporated into the dynactin complex. Using sucrose density gradient centrifugation, we showed that a significant proportion of GFP-Hsp150Glued is incorporated into the dynactin complex. Using sucrose density gradient centrifugation, we showed that a significant proportion of GFP-Hsp150Glued is incorporated into the dynactin complex. Using sucrose density gradient centrifugation, we showed that a significant proportion of GFP-Hsp150Glued is incorporated into the dynactin complex. Using sucrose density gradient centrifugation, we showed that a significant proportion of GFP-Hsp150Glued is incorporated into the dynactin complex. Using sucrose density gradient centrifugation, we showed that a significant proportion of GFP-Hsp150Glued is incorporated into the dynactin complex. Using sucrose density gradient centrifugation, we showed that a significant proportion of GFP-Hsp150Glued is incorporated into the dynactin complex.
Fig. 7. ER-to-Golgi transport is unperturbed in cells depleted of either lamin A/C, p150\textsuperscript{Glued}, CLIP-170 or EB1. (A) tsO45-G-YFP transport from the ER to Golgi was monitored by time-lapse microscopy. Cells were shifted to 32°C for 5 minutes before imaging. Bar, 10 μm. See supplementary material Movie 3. (B) Enlargements (2×) of the boxed regions (10×10 μm) in C show the presence of VTCs (arrows) that track to the Golgi (see supplementary material Movie 4). (C) Individual tsO45-G-YFP-labelled VTCs were tracked and grouped into bins of speeds of 0.2 μm s\textsuperscript{-1}. The number of objects in each bin were plotted for experiments in which lamin A/C (X), p150\textsuperscript{Glued} (●), EB1 (△) or CLIP-170 (○) had been depleted.

Fig. 8. Localization of human GFP-p150\textsuperscript{Glued} in HeLa cells. (A) GFP-Hsp150\textsuperscript{Glued} localizes to MT plus-ends in transiently transfected HeLa cells. (B) At intermediate expression level, p150\textsuperscript{Glued} decorates the length of MTs as well as localizing to punctate structures that can be seen in time-lapse sequences to translocate along MTs. Bar, 10 μm; arrows in inset highlight two structures that translocate in the direction of the arrows. See supplementary movie 5. (C) Tracking of GFP-p150\textsuperscript{Glued} in low expressing cells. Time-lapse imaging (see supplementary material Movie 7, upper panel) reveals punctate structures that translocate in a non-linear manner through the cytoplasm. Tracks are shown on the movie sequence that follow three such structures (supplementary material Movie 6, lower panel); one of these is overlayed on the still images taken from this movie (red). The lower panel shows a maximum-intensity projection of all time points from this sequence shows the trajectories taken by these structures as they translocate through the cytoplasm. (D) Density-gradient centrifugation shows that GFP-Hsp150\textsuperscript{Glued} co-distributes across the gradient with endogenous Hsp150\textsuperscript{Glued}. GFP and CLIP-170 distribute away from this complex whereas p50\textsuperscript{dynamitin} co-distributes as expected, with p150\textsuperscript{Glued}. We consistently observe a population of un-complexed p50\textsuperscript{dynamitin} for which we do not have a good explanation. (E) GFP-Hsp150\textsuperscript{Glued} puncta colocalize with ERGIC-53 (ER-Golgi transport intermediates, top panels), known to localize to dynamic structures, but not with COPII-coated (Sec24Cp-positive, lower panels) ER exit sites, which are mainly immobile. Arrowheads indicate colocalizing structures, arrows indicate structures that are only positive for GFP-Hsp150\textsuperscript{Glued}. 
consistently observed in these experiments and was not a result of GFP-Hsp150Glued expression (data not shown). Furthermore, a subset of GFP-Hsp150Glued punctate structures can be labelled with antibodies against ERGIC-53 (Fig. 8E, top panels), a protein that cycles between the ER and Golgi complex; anterograde ER-to-Golgi transport is known to require dynactin function (Presley et al., 1997). Not all GFP-Hsp150Glued structures are positive for ERGIC-53, consistent with its role in the movement of multiple types of intracellular membrane (Habermann et al., 2001). By contrast, no colocalization is seen with COPII, a marker for secretory cargo exit sites on the ER membrane (Fig. 8E, lower panels). These sites are relatively immobile (Stephens et al., 2000) and only show limited colocalization with endogenous p150Glued at steady-state (Watson et al., 2005).

Discussion

The major finding of our work is that plus-end localization of p150Glued is not required for diverse intracellular membrane trafficking events. These results also provide insight into the function of +TIPs in human cells. Our data demonstrate that, in mammalian cells, EB1 plays a role in the localization of CLIP-170 to MT plus-ends. This has been previously shown in fission yeast (Busch and Brunner, 2004) and Drosophila (Dzhindzhiev et al., 2005). Depletion of either EB1 or CLIP-170 causes a loss of p150Glued from plus-ends. The role of EB1 in this process might be through loading of CLIP-170 or by negatively regulating its dissociation (Komarova et al., 2005). CLIP-170 and p150Glued can interact independently of MT binding (Goodson et al., 2003) so it is possible that these two proteins are loaded together. Recent data support a functional role for the interaction of EB1 and p150Glued in which p150Glued acts as an allosteric activator of EB1 promoting microtubule assembly (Hayashi et al., 2005). Our data indicate that CLIP-170 has a significant role in this process in vivo. Plus-end tracking of CLIP-170 has recently been demonstrated to occur through pre-association with GTP-tubulin, co-polymerization and subsequent rapid dissociation (Folker et al., 2005). Our data would implicate EB1 in this loading step. This could explain how EB1 promotes rescue of MT polymerization following catastrophe (Askham et al., 2002; Ligon et al., 2003), and is also consistent with EB1 depletion resulting in a significant decrease in microtubule dynamics (Rogers et al., 2002) (our unpublished data). The precise role of EB1 and CLIP-170 in the localization of p150Glued to plus-ends is still unclear; they might in fact act in tandem, playing roles in both recruitment to the plus-end as well as in regulation of dissociation.

A recent study showed that, in Chinese hamster ovary cells, EB1 and EB3 together are required to maintain the localization of CLIP-170 at MT plus-ends (Komarova et al., 2005). In these cells, RNAi depletion of EB1 alone did not affect the localization of CLIP-170, whereas depletion of EB1 and EB3 together caused a significant decrease in CLIP-170 accumulation at plus-ends and (Komarova et al., 2005). We have not detected expression of EB3 in HeLa cells (see also http://symatlas.gnf.org/SymAtlas/) and our data suggest that, in HeLa cells at least, EB1 is sufficient to mediate this function. We suggest that the role of EB1/EB3 is to prevent dissociation of CLIP-170 from regions immediately adjacent to growing plus-ends (Komarova et al., 2005). Our data is consistent with this and cannot distinguish between roles for EB1 in loading, or preventing dissociation of, CLIP-170; this would largely depend on the mechanism of loading of EB1 and whether this is indeed restricted to the extreme tip of the MT (for example at a putative plus-end cap structure).

In our assays, depletion of EB1, CLIP-170 or p150Glued causes defects in the ability of cells to polarize in response to scratch wounding. It has previously been suggested that EB1 is not involved in MTOC reorientation (Wen et al., 2004). This was based on data showing that (1) overexpression of EB1-GFP does not promote MTOC reorientation and (2) that expression of EB1-C-GFP (amino acids 164-268 of EB1 fused to GFP) does not inhibit this process (Wen et al., 2004). However, there are concerns relating to the functionality of EB1-GFP when expressed in cells (Komarova et al., 2005), and despite acting as a dominant negative, presumably by binding to a crucial subset of interacting proteins, EB1-C-GFP does not affect the loading of endogenous EB1 onto MT plus-ends (Wen et al., 2004); therefore, it will not affect the plus-end localization of CLIP-170, nor the plus-end localization of p150Glued (Wen et al., 2004). It is likely that removal of CLIP-170 from plus-ends is primarily responsible for these defects (Fukata et al., 2002). However, the effects of depleting p150Glued itself suggests that the loading of p150Glued onto plus-ends is required for some aspects of polarization.

We have shown that a significant proportion of GFP-Hsp150Glued becomes incorporated into the dynactin complex when expressed transiently in HeLa cells at low levels. GFP-Hsp150Glued also localizes to small, highly dynamic punctate structures that translocate along MTs through the cytoplasm. Although we cannot directly correlate these pools of protein, these data are consistent with GFP-Hsp150Glued being associated with highly mobile intracellular membranes as part of the dynactin complex. A similar observation has been made for the Arabidopsis orthologue of EB1 (Mathur et al., 2003); punctate GFP-AtEB1 structures were detected and shown to localize with markers for ER membranes. However, we have not detected any similar structures with human EB1. At higher levels of expression, GFP-Hsp150Glued decorates microtubules. This is also seen with p150Glued orthologues when overexpressed but is not seen with antibody labelling (Quintyne et al., 1999; Vaughan et al., 2002; Waterman-Storer et al., 1995). This therefore probably represents an artefact of overexpression but might reflect that fact that the CAP-Gly motif of p150Glued itself can bind along the length, not just to the plus-end, of MTs.

Despite significant defects in other cellular processes, we detected no perturbation of intracellular traffic following depletion of cells with these three +TIPs. It is somewhat surprising that we do not observe any decrease in endolysosomal motility in cells depleted of CLIP-170; there is significant evidence in favour of a role for CLIP-170 in endosome motility (Pierre et al., 1992; Valetti et al., 1999). Since RNAi-mediated depletion is not complete, our work does not formally rule out a function for CLIP-170 in the endocytic pathway at sites other than MT plus-ends. The same is true for EB1 and p150Glued. However, our data clearly show that the localization of p150Glued to MT plus-ends is not required for these membrane trafficking steps. Consistent with this, ultrastructural localization of p150Glued at plus-ends did not show any association with organelles (Habermann et al., 2001). Our data is also consistent with that showing that CLIP-170...
knockout mice have few obvious defects other than in spermatogenesis (Akhanova et al., 2005).

It is somewhat surprising that we do not observe any significant defects in membrane traffic following depletion of p150\textsuperscript{Glued} itself. Dynactin has been implicated in a diversity of membrane trafficking steps and yet we observe only a minor relocation of organelles that are normally clustered in a juxtanuclear localization to a more peripheral location. The simplest explanation for this is that we are only depleting p150\textsuperscript{Glued} to around 50% of normal levels, suggesting that it is not limiting for normal intracellular transport. Intriguingly, despite this partial depletion of the protein, we observe a near-complete loss of p150\textsuperscript{Glued} immuno-reactivity from MT plus ends. One explanation for this is that the plus-end is one of the lower affinity binding sites for dynactin and yet it is this pool that is preferentially detected using the monoclonal antibody used in this study.

In summary, a loss of p150\textsuperscript{Glued} from plus-ends, following depletion of either EB1 or CLIP-170, does not cause any detectable defects in membrane traffic. Although there is significant and highly convincing data regarding the capture of kinetochores during mitosis (Biggins and Walczak, 2003; Dzhindzhev et al., 2005; Mimori-Kiyosue and Tsukita, 2003), and capture mechanisms at the cell cortex (Busch and Brunner, 2004; Fukata et al., 2002; Mimori-Kiyosue and Tsukita, 2003), our data are not consistent with membrane-microtubule ‘search-and-capture’ mechanisms involving a plus-end pool dynactin (Vaughan, 2005; Vaughan et al., 2002; Watson et al., 2005). From our data, we conclude that these +TIPs are recruited to membranes independent of their interaction with MT plus-ends. Multiple adaptors are implicated in the recruitment of motors and their accessory proteins to membranes (reviewed in Karcher et al., 2002). Potential mechanisms include Rab6-dependent recruitment of dynactin to Golgi membranes (Short et al., 2002) — probably via an interaction with the dynactin-interacting protein Bicaudal-D (Matanis et al., 2002) — and Rab7-dependent recruitment of dynactin to lysosomes (Jordens et al., 2001). Other examples include a role for ZW10 in ER-to-Golgi transport (Hirose et al., 2004), and the Cdc42-dependent recruitment of dynein to COPI (coatamer) coated membranes (Chen et al., 2005). It will be important to define the precise molecular detail of how these complexes are formed and how they are regulated to control intracellular membrane movement.

Materials and Methods

HeLa cells (ATCC CCL-2) were grown on glass-bottom dishes (MatTek Corp., Ashland, MA) or 22 mm coverslips in DMEM (Life Technologies, Karlsruhe, Germany) supplemented with 10% FCS and 1% glutamine. Monoclonal mouse anti α-tubulin (DM1A) was from Neomarkers (Fremont, CA), anti-giantin from Covance (Harrogate, UK), and pericentrin (ab4448) from Abcam (Cambridge, UK). Monoclonal antibodies directed against p50\textsuperscript{dynamitin} (610003), EB1 (610534) and p150\textsuperscript{Glued} (610474 and 612709) were from Pharmingen (BD Biosciences, San Diego, CA), T. E. Kreis) was from Harry Mellor (University of Bristol, Bristol, UK), and pericentrin (ab4448) from Abcam (Cambridge, UK). Monoclonal antibodies directed against p50\textsuperscript{dynamitin} (610003), EB1 (610534) and p150\textsuperscript{Glued} (610474 and 612709) were from Pharmingen (BD Biosciences, San Diego, CA), T. E. Kreis) was from Harry Mellor (University of Bristol, Bristol, UK), and pericentrin (ab4448) from Abcam (Cambridge, UK). Monoclonal antibodies directed against p50\textsuperscript{dynamitin} (610003), EB1 (610534) and p150\textsuperscript{Glued} (610474 and 612709) were from Pharmingen (BD Biosciences, San Diego, CA), T. E. Kreis) was from Harry Mellor (University of Bristol, Bristol, UK), and pericentrin (ab4448) from Abcam (Cambridge, UK). Monoclonal antibodies directed against p50\textsuperscript{dynamitin} (610003), EB1 (610534) and p150\textsuperscript{Glued} (610474 and 612709) were from Pharmingen (BD Biosciences, San Diego, CA), T. E. Kreis) was from Harry Mellor (University of Bristol, Bristol, UK), and pericentrin (ab4448) from Abcam (Cambridge, UK). Monoclonal antibodies directed against p50\textsuperscript{dynamitin} (610003), EB1 (610534) and p150\textsuperscript{Glued} (610474 and 612709) were from Pharmingen (BD Biosciences, San Diego, CA), T. E. Kreis) was from Harry Mellor (University of Bristol, Bristol, UK), and pericentrin (ab4448) from Abcam (Cambridge, UK).

Immunofluorescence and live cell imaging

Living and fixed cells were imaged by using wide-field microscopy as previously described (Stephens, 2003). For immunofluorescence, cells were fixed with methanol at −20°C for 4 minutes, blocked using PBS containing 3% bovine serum albumin and incubated with primary antibodies for 1 hour. Secondary antibodies labelled with Alexa Fluor 488, Alexa Fluor 568, or Alexa Fluor 647 were from Invitrogen (Alexa Fluor 568-transferrin (Molecular Probes/Invitrogen, Paisley, UK) was diluted in growth medium and added to cells at a final concentration of 10 μg ml⁻¹. Images and time-lapse sequences were acquired 10 minutes after Alexa Fluor 568-transferrin incubation. Wide-field images were acquired with a monochromator-based wide-field imaging system (TILL Photonics, Martinsried, Germany). From our data, we conclude that these +TIPs are recruited to membranes independent of their interaction with MT plus-ends. Multiple adaptors are implicated in the recruitment of motors and their accessory proteins to membranes (reviewed in Karcher et al., 2002). Potential mechanisms include Rab6-dependent recruitment of dynactin to Golgi membranes (Short et al., 2002) — probably via an interaction with the dynactin-interacting protein Bicaudal-D (Matanis et al., 2002) — and Rab7-dependent recruitment of dynactin to lysosomes (Jordens et al., 2001). Other examples include a role for ZW10 in ER-to-Golgi transport (Hirose et al., 2004), and the Cdc42-dependent recruitment of dynein to COPI (coatamer) coated membranes (Chen et al., 2005). It will be important to define the precise molecular detail of how these complexes are formed and how they are regulated to control intracellular membrane movement.

Quantification of plus-ends p150\textsuperscript{Glued}

To quantify plus-end p150\textsuperscript{Glued}, cells were fixed and immunolabelled with mouse monoclonal antibodies against α-tubulin and p150\textsuperscript{Glued}. 1 μg of antibody was combined with 5 μl of Zenon mouse IgG labelling reagent and incubated for 5 minutes at room temperature. 5 μl of Zenon blocking reagent was then added and carefully mixed and incubated for 5 minutes at room temperature. The complex was used immediately. 12-bit images acquired by using wide-field microscopy were thresholded (250-500 grey levels) to remove background fluorescence and retain plus-end labelling within the linear range. Average pixel intensities were calculated along a 10-pixel (1.34 μm) line originating from the end of the microtubule (as detected by tubulin fluorescence) using ImageJ. Immunofluorescence processing protocols and imaging exposures times and processing were constant between samples to allow quantification.

Anterograde transport assays

Cells were depleted of +TIPs by using siRNA transfection (60 hours incubation) followed by transfection with a plasmid encoding NA-GFP (Shima et al., 1997) and incubation for a further 24 hours. Cells were incubated for 2 hours in 5 μg ml⁻¹ brefeldin A followed by extensive washing for 5 minutes and time-lapse imaging. tsO45-YFP was expressed either from plasmid DNA (Toomre et al., 2001). No difference in transport was observed using either method of expression.

Scratch wounded

A549 cells were grown on glass-bottom dishes (MatTek) and transfected with the same sites in pEGFP-C1 (Clontech). Accuracy of the GFP clone was confirmed by DNA sequencing (MWG Biotech) and comparison to the published sequence. Two clones to the published sequence were identified (A3195T and A3393T) neither of which affects the protein sequence. This clone differs in two positions to an alternative human p150\textsuperscript{Glued} clone in the GenBank database (accession NM_004082) by having glycine instead of glutamic acid at positions 577 and 1011 of the protein sequence.

RNAi and transfection

siRNA duplexes CLIP-170a and CLIP-170b have been previously described (Lansbergen et al., 2004); other duplexes were designed using the online algorithms of, and subsequently synthesized by, MWG-Biotech. Duplexes were BLAST searched against the non-redundant database to determine specificity. Sequence alignment was performed with DNASTar (Madison, USA). Sequences used were as follows: CLIP-170 a: GGA GAA GCA GCA GCA CAU A; CLIP-170 b: UGA AGA UGU CAG GAG AUA A; CLIP-170 c: GCA GAA GUA GAA GAC A; CLIP-170 d: GAA GUC AGU AGU CAG GAG AUA A; EB1 a: AUU CCA AGC UAA GCC AGA ATT; EB1 b: UUC GUU CAG UGG UUC AAG ATT; p150\textsuperscript{Glued} a: GUA CUU CAC UUG UGA UGA ATT; p150\textsuperscript{Glued} b: GUA CAG GAC AUA AAU ATT; lamin A/C: CUG GAC UUC UAC AAG AAC A.

Cells were transfected by using the calcium phosphate method at 3% CO₂ (Chen and Okayama, 1988); the medium was changed 16 hours after transfection and cells were transfected to 5% CO₂ before being assayed at the times indicated. All times are from the point of addition of calcium phosphate precipitates, i.e. including this 16 hour incubation.
siRNA duplexes by using calcium phosphate. 72 hours after transfection, cell monolayers were wounded using a 200 μl micropipette tip and incubated for a further 2 hours in complete growth medium containing 10% fetal calf serum. Cells were then fixed and processed for immunofluorescence as described above. Cell polarization in response to wounding was analyzed using Velocity 3.5 (Improvision) to mark the cell centroid followed by overlay of the template onto this centroid. The presence of the Golgi (giantin localization) or centrosome (pericentrum) was determined for at least 100 cells from each of three independent experiments.

Sucrose density gradient centrifugation
Sucrose density gradient centrifugation was performed as described in Watson et al. (Watson et al., 2005).

References

Akhananova, A., Mausset-Bonnefont, A. L., van Cappellen, W., Keijzer, N., Hoogenaar, C. C., Stepanova, T., Drabek, K., van der Wees, J., Moommaas, M., Onderwater, J. et al. (2005). The microtubule plus-end-tracking protein CLIP-170 associates with the spermatid manchette and is essential for spermatogenesis. *Genes Dev.* 19, 2501-2515.

Aridor, M., Bannykh, S. L., Rowe, T. and Balch, W. E. (1995). Sequential coupling between COPI and COPII vesicle coats in endoplasmic reticulum to Golgi transport. *J. Cell Biol.* 131, 875-893.

Ashkan, J. M., Vaughan, K. T., Goodman, H. V. and Morrison, E. E. (2002). Evidence that an interaction between EB1 and p150(Glued) is required for the formation and maintenance of a radial microtubule array anchored at the centrosome. *Mol. Biol. Cell.* 13, 3627-3645.

Berrueta, L., Tirnauer, J. S., Schuyler, S. C., Pellman, D. and Bierer, B. E. (1999). Bicaudal-D regulates COPI-ERG-Golgi transport by recruiting the dynein-dynactin motor complex. *Nat. Cell Biol.* 1, 89-97.

Bercovitz, A., Schroer, T. A., Griffiths, G. and Burkhardt, J. K. (2001). Immunolocalization of cytoplasmic dynein and dynactin subunits in cultured macrophages: enrichment on early endocytic organelles. *J. Cell Sci.* 114, 229-240.

Hayashi, I., Wilde, A., Mai, T. K. and Ikura, M. (2005). Structural basis for the activation of microtubule assembly by the EB1 and p150(Glued) complex. *Mol. Biol. Cell.* 16, 449-460.

Hirose, H., Arasaki, K., Dohmae, N., Takio, K., Hatusuza, K., Nagahama, M., Tani, K., Yamamoto, A., Tohyama, M. and Tagaya, M. (2004). Implication of ZW10 in membrane trafficking between the endoplasmic reticulum and Golgi. *EMBO J.* 23, 1276-1278.

Jordens, I., Fernandez-Borja, M., Marsman, S., Dusseljee, S., Janssen, L., Calafat, J., Janssen, H., Wubbolts, R. and Neefjes, J. J. (2001). The Rab7 effector protein RILP controls lysosomal transport by inducing the recruitment of dynein-dynactin motors. *Curr. Biol.* 11, 1680-1685.

Karcher, R. L., Deacon, S. W. and Gelfand, V. I. (2002). Motor-cargo interactions: the key to transport specificity. *Trends Cell Biol.* 12, 21-27.

Keller, P., Toomre, D., Diaz, E., White, J. and Simons, K. (2001). Multicolour imaging of post-Golgi sorting and trafficking in live cells. *Nat. Cell Biol.* 3, 140-149.

Kumar, L., Lamsberg, G., Galjart, N., Grosfeld, F., Borisy, G. and Akhananova, A. (2005). EB1 and EB3 control CLIP dissociation from the ends of growing microtubules. *Mol. Biol. Cell.* 16, 5334-5345.

Lansbergen, G., Komarova, Y., Modesti, M., Wymann, C., Hoogenaar, C. C., Goodman, H. V., Lamert, R. P., Drefchel, D. N., Van Munster, E., Gudella, T. W., Jr et al. (2004). Conformational changes in CLIP-170 regulate its binding to microtubules and dynactin localization. *J. Cell Biol.* 166, 1003-1014.

Ligon, L. A., Shelly, S. S., Tokito, M. and Holzhauser, E. L. (2003). The microtubule plus-end proteins EB1 and dynactin have differential effects on microtubule polymerization. *Mol. Biol. Cell.* 14, 1405-1417.

Matsuishi, T., Akhananova, A., Wolf, P., Del Nery, E., Weide, T., Stepanova, T., Galjart, N., Grosfeld, F., Goud, B., Evers, C. L. et al. (2002). Bicaudal-D regulates complete Golgi-ER transport by recruiting the dynein-dynactin motor complex. *Nat. Cell Biol.* 4, 986-992.

Mather, J., Mathur, N., Kernebeck, B., Sinivas, B. P. and Hulskamp, M. (2003). A novel localization pattern for an EB1-like protein links microtubule dynamics to endomembrane organization. *Curr. Biol.* 13, 1991-1997.

Mimori-Kiyosue, Y. and Tsukita, S. (2003). “Search-and-capture” of microtubules through plus-end-binding proteins (+TIPS). *J. Biochem.* 134, 321-326.

Mimori-Kiyosue, Y., Onderwater, J. et al. (Watson et al., 2005).

Moro, G., Schroer, T. A., Strickland, S. I., Galli, M. et al. (2004). Implication of ZW10 in membrane trafficking between the endoplasmic reticulum and Golgi. *Mol. Biol. Cell.* 16, 1137-1148.

Monteiro, J. M., Vaughan, K. T., Goodman, H. V. and Morrison, E. E. (2002). The Rab6 GTPase regulates recruitment of the dynactin complex to Golgi membranes. *Curr. Biol.* 12, 1792-1795.

Mori, S., Kato, M., Nishimura, S., Kondo, H., Sato, Y. and Endo, Y. (2000). Bicaudal-D regulates COPI-ERG-Golgi transport complexes segregate from COPI in close proximity to exocytic sites. *J. Cell Sci.* 113, 2177-2185.

Onderwater, J. et al. (Watson et al., 2005).

Peters, M. H., Koolman, H., Wubbolts, R. and Neefjes, J. J., Janssen, H., Wubbolts, R. and Neefjes, J. J. (2001). The Rab7 effector protein RILP controls lysosomal transport by inducing the recruitment of dynein-dynactin motors. *Curr. Biol.* 11, 1680-1685.

Wallar, B. J., Alberts, A. S. and Gundersen, G. G. (1991). Dynactin, a conserved, ubiquitously expressed component of an activator of cytoplasmic dynein. *Curr. Biol.* 1, 887-900.

Watson, P., Forster, R., Palmer, K. J., Pepperkok, R. and Stephens, D. J. (2005). Evidence for regulated binding of p150(Glued) to microtubule plus ends. *Nature Struct. Mol. Biol.* 12, 986-992.

Waterman-Storer, C. M., Karki, S. and Holzbaur, E. L. (1999). ER-to-Golgi transport visualized in living cells. *Nature* 398, 81-85.

Watson, P., Forster, R., Palmer, K. J., Pepperkok, R. and Stephens, D. J. (2005). Evidence for regulated binding of p150(Glued) to microtubule plus ends. *Nature Struct. Mol. Biol.* 12, 986-992.

Waterman-Storer, C. M., Karki, S. and Holzbaur, E. L. (1999). ER-to-Golgi transport visualized in living cells. *Nature Struct. Mol. Biol.* 12, 986-992.

Waterman-Storer, C. M., Karki, S. and Holzbaur, E. L. (1999). ER-to-Golgi transport visualized in living cells. *Nature Struct. Mol. Biol.* 12, 986-992.

Waterman-Storer, C. M., Karki, S. and Holzbaur, E. L. (1999). ER-to-Golgi transport visualized in living cells. *Nature Struct. Mol. Biol.* 12, 986-992.