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# Review

# Action and interactions at microtubule ends

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**Abstract.** Microtubule dynamic instability is fundamentally important to the way cells respond to their environment and segregate their genetic material. A disparate class of proteins defined by their localisation to growing microtubule plus ends ('+TIPS') play a key role in controlling microtubule dynamics and organisation. They directly impact upon the behaviour of the microtubule tip and link this structure to interfaces that include kinetochores and the cortex of the cell. Surprisingly, some +TIPs also have important functions at the microtubule minus end. These properties contribute to the important roles played by +TIPs in processes such as mitosis and cell migration. This review examines how recent advances have impacted our understanding of +TIP function in mammalian cells, with emphasis on the emergence of the EB1 family as a core component of +TIP activities. An overview of the use of +TIP imaging as a tool for the cell biologist is also presented.

**Keywords.** +TIP, microtubule, dynamics, migration, mitosis.

## Introduction

Microtubules (MTs) are dynamic biological polymers essential for eukaryotic life. They are the main structural component of the mitotic spindle, while in interphase or post-mitotic cells they serve as structural supports, highways for the trafficking of cargo by MT-associated motors and templates for the distribution of intracellular organelles. MTs are a lattice assembled from heterodimers of $\alpha$-$\beta$ tubulin, which endows them with an intrinsic polarity. MTs assembled from purified tubulin *in vitro* grow faster at one end than the other; this is the end terminated by $\beta$-tubulin and it is referred to as the MT plus end. Conversely, the slower growing end terminated by $\alpha$-tubulin is called the minus end. Within cells most MT growth occurs at the plus end of the polymer, while the minus end is usually stably capped and often anchored at a specialised structure called the centrosome, which is also the organelle responsible for the nucleation of new MTs. MT plus ends exhibit a behaviour called dynamic instability; they can quickly switch between phases of growth (where tubulin heterodimer is added to the end of the lattice), rapid shrinkage (where heterodimer is lost from the end of the lattice) or pause (where no net gain or loss of heterodimer occurs). Two further descriptive terms, catastrophe (a direct transition from growth to shrinkage) and rescue (a direct transition from shrinkage to growth), allow the dynamic behaviour of a MT plus end to be completely and formally described [1].

Dynamic instability is an intrinsic feature of MTs and can be observed in purified preparations *in vitro*. MTs within cells display similar behaviours, but the specific parameters of dynamic instability are influenced by a variety of MT-associated proteins (MAPs) that allow the cell to assemble and modify an extensive MT network. One class of MAPs are defined by their specific localisation to growing MT plus ends (Fig. 1). Called '+TIPs', their identification and study is intimately linked to the revolution in intracellular imaging driven by the development of fluorescent fusion proteins and digital imaging technology. Indeed, the entire +TIP field owes its existence to modern imaging techniques since they are one of the

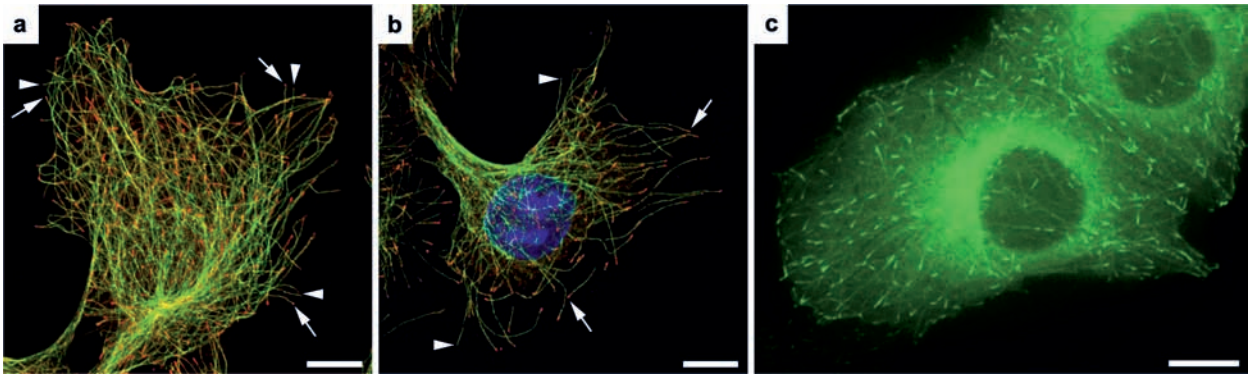


**Figure 1.** +TIPs are defined by their localisation and dynamics. (*a*) COS-7 cell co-immunostained for tubulin to reveal MTs (green) and the +TIP protein EB1 (red). EB1 is present at the end of some MTs (arrows) but not others (arrowheads). In these cells the great majority of MT ends at the cell periphery are plus ends. (*b*) An MDCK cell scattering in response to HGF was coimmunostained for MTs (green) and EB1 (red). MDCK cells contain a MT array that is not anchored at the centrosome. EB1 can be seen to localise to only one end of free cytoplasmic MTs (arrows). (*c*) One frame from a time-lapse movie of a COS-7 cell expressing EB3-GFP. The 'comet'-like distribution typical of +TIPs is clearly visible. Note also the background fluorescence from free cytoplasmic protein that has largely been extracted by the methanol fixation used in panels *a* and *b*. Analysis of movies such as this confirmed the specific localisation of +TIPs to growing MT plus ends. Bars, 10 µm.

few classes of protein defined by their localisation and dynamic behaviour rather than, for example, a common structural motif. In fact, the +TIP 'family' is a disparate group, containing MT motor complex components, signal transduction molecules and molecular adaptors.

MT plus end binding proteins are present in all eukaryotes. Defining the functions of +TIPs has proved challenging, since researchers are probing the localised, transient and promiscuous interactions of a variable complement of proteins associated with a motile structure that is itself exquisitely sensitive to perturbation. Nevertheless, progress has been impressive, and a substantial literature has been generated in the relatively short time since the first +TIP was described [2]. This review will focus upon the functional interactions of mammalian +TIPs. In particular, the properties of the EB1 protein family will be examined since they are now emerging as a key player in the functions of this class of MAPs as a whole. Finally, an overview of the use of +TIPs as experimental tools in mammalian systems will be presented.

## Angels dancing on the head of a pin: just how many +TIPs are there?

At first glance an unfeasibly large (and constantly increasing) number of proteins seem to track growing MT ends (Table 1). Terminal congestion at MT tips appears to be avoided in part by tissue-specific expression patterns, with direct competition for a finite number of binding sites and divergent methods of association with MT tips also playing roles in limiting the specific complement of +TIPs on an individual MT end [3–6]. It seems likely that this collection of proteins will eventually be

**Table 1.** Properties of mammalian +TIPs.

| Mammalian +TIP | Direct binding to growing MT tip? | Obligate MT tip binder? | Direct mammalian EB1 interaction? |
|---|---|---|---|
| EB family | yes | yes | N/A |
| CLIP-170, -115 | yes | yes | yes |
| p150Glued | yes | yes | yes |
| ACF7 (MACF) | not reported | no | yes |
| APC | not reported | no | yes |
| chTOGp | ? | ? | not reported |
| CLASP1, 2 | yes | no | yes |
| LIS1 | not reported | no | not reported |
| MCAK | ? | ? | not reported |
| mNav1 | possibly | possibly | not reported |

Currently, direct binding to growing MT ends is accepted for proteins in the EB family and +TIPs with a CAP-Gly MT interaction domain. A localisation to all growing MT tips *in vivo* is seen for the same set of proteins minus the CLASPs where regionally regulated tip-tracking has been proven. The chTOGp listing contains question marks because binding to MT ends has been seen *in vitro*, but a localisation to growing tips *in vivo* has not been reported. The MCAK listing contains question marks since its direct MT end binding is likely to relate to its MT depolymerisation activity, and although the protein may be found at all growing tips *in vivo*, this may therefore represent indirect binding. 'Possibly' in the mNav1 listing reflects both the lack of published information on this protein and the possibility that, since it contains a genuine MT association domain and coimmunoprecipitates with tubulin, it might be the first member of a new family of direct and obligate binders of growing MT tips. 'Not reported' should not be taken as an indication that the property has been tested for the protein in question.

separable into at least two subclasses, those that directly recognise and associate with growing MT plus ends and those that interact with this core group. The first subclass might also be separable into those proteins that are obligated to associate with all growing MT plus ends in a cell and those which target only a subset of MT ends. At present, however, given the observations that +TIP interactions with each another are extensive, that many +TIPs contain domains capable of directly interacting with MTs and that the most distinctive +TIP behaviour (a dynamic association with growing MT ends) has so far stubbornly resisted replication in defined *in vitro* experimental systems (though see [7] for recent progress in this area), this differentiation remains incomplete. Nevertheless, the key players at the MT end are likely to have now been identified (Table 1). The prototypic +TIP is CLIP-170 [2], and the closely related protein CLIP-115 [8] shares its tip-tracking ability. Three members of another protein family, EB1, EB2 (RP1) and EB3 are all +TIPs [9–12]. The +TIP collective also includes the dynein/dynactin microtubule motor complex, in particular the p150Glued subunit of dynactin [13, 14]; CLASP1 and CLASP2 [15]; the spectroplakin ACF7 (MACF) [16]; LIS1 [17]; the adenomatous polyposis coli (APC) tumour suppressor protein [18, 19]; the Kin I kinesin MCAK [20]; the myosin V-associated protein melanophilin [21]; and the relatively uncharacterised mNav1, a protein highly expressed during development of the mouse nervous system that may represent the first member of a new subclass of +TIPs [22]. Definitive proof of tip-tracking behaviour for the mammalian chTOGp protein [23, 24] is currently lacking, but the protein is found in most +TIP listings since it acts at this site [25] and a MT tip localisation has been demonstrated for its orthologues in *Drosophila* and *Dictyostelium* [26– 28]. Of the above proteins, the EB family, the CLIPs and p150Glued are found on all growing MT ends and have the best claim to be core components of +TIP complexes.

## The microtubule plus end

MT polymerisation is an energy-dependent process. Tubulin possesses an intrinsic GTPase activity and GTP hydrolysis provides the energy for MT growth. This hydrolysis appears to be closely associated with structural changes at the MT tip. GTP tubulin has a high affinity for the MT plus end and polymer terminating in GTP tubulin is resistant to depolymerisation. Once added to the lattice, the $\beta$ subunit in the newly assimilated heterodimer lacks structural determinants needed to hydrolyse its bound GTP. These determinants are provided by an interaction with the $\alpha$-subunit of the next heterodimer added to the polymer. The subsequent hydrolysis of GTP induces curvature in the polymer, so the structure of the GTP-bound lattice at the MT plus end is different from that of the GDP-bound lattice forming the main body of the polymer, resembling a flat sheet rather than a tube (see [4] for a succinct overview of this topic). This structural difference may provide +TIPs with the means of achieving their polarised distribution on the MT polymer. It should also be evident that proteins that specifically associate with the MT plus end are in the ideal location to either influence MT dynamics or interact with targets encountered by the dynamic MT plus end.

## How do +TIPs recognise a growing MT plus end?

Remarkably, the mechanisms used by obligate +TIPs such as EB1 or CLIP-170 to achieve their localisation to growing MT ends in mammalian cells have yet to be fully defined. It is clear that +TIPs have a higher affinity for the growing MT plus end than the barrel of the MT [29] and in the case of +TIPs containing a CAP-Gly (cytoskeleton-associated protein with a conserved glycine) microtubule interaction domain (for example, CLIPs, CLASPs and p150Glued), a strong preference for microtubule polymer composed of heterodimer containing tyrosinated $\alpha$-tubulin [30]. The same is not true for EB proteins. However, $\alpha$-tubulin mutagenesis and two-hybrid interaction studies in budding yeast indicate that tubulin binding by the EB orthologue Bim1p is abolished by mutations clustered in a region predicted to be oriented outwards in an assembled MT [31]. Interestingly, other mutations that abrogated Bim1p binding were located at the intradimer interface, consistent with the idea that an EB protein interaction with $\alpha$-tubulin requires it to be part of an $\alpha$–$\beta$ heterodimer.

It has also been noted that GFP/+TIP fusion proteins that specifically target growing MT ends at low expression levels in transfected cells acquire an unpolarised distribution along the MT as expression levels rise, consistent with the concept of binding to low-affinity sites on the MT barrel once high-affinity sites at growing MT tips are saturated (i.e. [32]). More recently, fluorescence speckle microscopy has indicated that +TIPs associate with the newly polymerised lattice, where they remain stably bound until a release from older polymer is observed [33, 34]. This ‘treadmilling’ provides a kinetic basis for the comet-like appearance of +TIPs on MT ends. Recent work suggests that for CLIP-170, this initial association might arise by copolymerisation of +TIPs with tubulin heterodimer into the growing MT end [7, 33–35], consistent with the observation that CLIP-170 has a higher affinity for tubulin heterodimer than for MTs assembled from GDP or GTP-tubulin in *in vitro* assays [34]. Nevertheless, no study has yet been able to definitively exclude the possibility that the classic +TIP distribution is a consequence of binding to a tubulin conformation, such as

flat sheets of tubulin polymer [36, 37], that is only present at the growing MT plus end. This idea is attractive as it provides a mechanism for the dissociation of +TIPs from older MT polymer, where release is triggered by conformational change when the sheet curves into a tube. Of course, the two models are not mutually exclusive, and a model where +TIPs bind to tubulin heterodimer with high affinity in a manner that is unaffected by incorporation into a flat lattice but is antagonised by the transition to a curved lattice seems possible.

A consequence of the above models is that the +TIP comets seen during imaging represent a transiently immobilised population of protein visible against the background of a freely diffusible cytoplasmic pool. An implication of this is that any interaction with a +TIP on a growing MT end must therefore also be transient. In the case of an MT undergoing a period of steady growth, if average comet length is around 1 μm (i.e. [2, 38]) and the average speed of MT growth is around 20 μm/min [2, 11, 14, 15 etc.] then an interaction between an individual +TIP molecule and its ligand at this site can only last for around 3 s. On the other hand, treadmilling ensures that there is always a new +TIP available to bind to nearby as the MT grows. It should also be noted that several studies have reported an EB1 association with the tip of stable, cortically captured microtubules [39, 40]. This observation is so far restricted to fixed and immunostained cells examined by total internal reflection fluorescence microscopy, and confirmation in living systems would be desirable. The observations are intriguing since it seems possible that they might reflect the nature of the stabilisation of the captured MTs which, rather than being truly paused, might be undergoing transient and highly spatially restricted periods of dynamic behaviour undetectable by conventional fluorescence microscopy.

## Interactions between +TIPs: Is EB1 the universal MT plus end binding protein?

One reason why defining tip-tracking mechanisms has proven so difficult is that extensive interactions between +TIPs have been identified, raising the possibility that at least some of the observed *in vivo* localisations at MT ends could reflect 'piggy-backing' of one +TIP on another. In fact, the situation is even more complicated than this.

As a rule of thumb one might do well to assume that any single +TIP associated with all other proteins that act at MT plus ends until otherwise proven. Whether these associations are direct or indirect, cooperative or competitive and if they have implications for the functional activities of individual +TIPs are questions that have occupied researchers for the last few years. An outcome of these studies is the realisation that one +TIP, EB1, is often a common factor in the growing web of functional interactions (Table 1) [7, 16, 21, 32, 33, 38, 41–48]. Of all the +TIPs listed in this review, EB1 has *not* been shown to directly interact with LIS1 or MCAK in at least one experimental system (though see [49] for an EB1-kinesin 13 interaction in *Drosophila* that might be of interest in the context of mammalian MCAK tip-tracking). EB1 is a small protein, though it can exist as a dimer, and it has a simple domain organisation. Its structure has been extensively studied [44, 47, 48, 50]. In the N-terminal half of the protein a calponin homology (CH) domain mediates binding to MTs and potentially tubulin heterodimer [32, 50]. EB1 deletion mutants lacking this domain form aggregates in transfected cells while retaining the ability to interact with ligands such as APC, suggesting that EB1 is composed of two functional domains separated by a central linker region [51]. The C-terminal half of the protein harbours both its dimerisation region and the interaction sites for all ligands so far identified [32, 44–48]. Given its size it is unsurprising that the binding of different EB1 ligands seems to be mutually exclusive (i.e. [7, 32]). Interestingly, EB1 terminates in an acidic tail similar to the 'E-hook' present in $\alpha$-tubulin [33], a region that appears to play an important role in many of its interactions [32, 33, 46, 48]. As noted above, three EB proteins have been identified in humans. Where investigated it appears that interactions identified for EB1 can be replicated by EB3 but not RP1 [46]. The latter is the most divergent family member and remains comparatively undefined in comparison to its siblings [12, 46, 52, 53]. Overall, vertebrate EB proteins appear to promote MT growth and stability by decreasing catastrophe frequencies [29].

EB1 was first identified in a yeast-2-hybrid screen for proteins that interacted with the C-terminal region of APC [41]. For a time, therefore, EB1 was regarded as an accessory to APC function. The identification of other interactions for EB1 increased interest in the protein, while recent findings may even have reversed the pecking order between the two molecules, at least with regard to effects upon MT growth. Previous work had demonstrated that EB1 had an ability to promote MT growth *in vitro* only when it was associated with an APC fragment containing its interaction site [54]. Hayashi and colleagues, investigating the p150Glued interaction with EB1, recently provided a structural explanation for this observation [48]. When ligands bind to the EB1 C-terminal region, they relieve an auto-inhibitory conformation that allows the protein to associate with MT ends and promote MT growth [48]. Ligand binding therefore appears to be a prerequisite for EB1 function at MT tips. So, rather than viewing EB1 as an accessory to APC function, it may be more useful to regard APC as one of many cofactors that competitively activate EB1 activity and hence promote MT growth and stability in specific cellular regions,

though an independent role for APC in MT stabilisation also seems likely [38, 55–58]. The relief of self-inhibitory protein conformations may prove to be a common theme among +TIPs, since CLIP-170 binding to MTs seems to be similarly regulated [59].

Another observation from the study by Hayashi and colleagues [48] serves to illustrate the complexity of +TIP interactions. The EB1 binding region in p150Glued overlaps with the MT binding CAP-Gly domain in the latter protein, inhibiting its function. The implication of this is that at least some portion of the p150Glued present on MT ends may be there courtesy of EB1 (activating the MT growth-promoting activity of EB1 at the same time) rather than via direct binding to the MT end. A similar interaction might help target other CAP-Gly containing +TIPs to growing MT ends. However, in the case of the CLIP proteins it has been suggested that they either bind to MT ends independently of EB1 and that the latter suppresses their too-rapid dissociation from the growing tip [33], or that the interaction with EB1 unfolds CLIP-170 and licences it for copolymerisation with tubulin into MTs [7], thereby contributing to the targeting of EB1 to MT ends at the same time. Unravelling whether any of these possibilities predominate *in vivo*, and how EB/CAP-Gly protein/tubulin complexes are organised, should prove challenging. However, from an EB1-centric viewpoint it is worth noting that APC C-terminal fragments that localise to MT ends in transfected cells (presumably by unfolding EB1 and simultaneously blocking any interaction with CAP-Gly proteins [32]) need not contain an MT binding site [38]. Furthermore, in mammalian cells an EB1 MT plus end localisation is maintained in the absence of APC, CLASPs, CLIPs, p150Glued and spectroplakins [i.e. 9, 15, 16, 60]. These findings indicate that an interaction with another MAP or any specific CAP-Gly containing +TIP is not necessary for EB1 tip tracking, but imply that its unfolding by ligand binding is essential. A prediction of this hypothesis is that competitive inhibition of EB1 ligand binding should remove EB1 (and its growth promoting activity) from MT ends. This should be experimentally testable.

Within cells EB1 appears to be an unavoidable and universal promoter of MT plus end growth. It is present and active at every growing MT tip, and it can be inferred that tip-tracking EB1 is always bound to a ligand. As such, EB proteins may prove to be the core component of the +TIP complement at a growing MT end.

## Roles for EB1 in cell migration

Further support for the core importance of EB1 at MT plus ends is that it has been implicated in a range of processes that may act independently in coordinating directed cell migration. An excellent review has recently covered this topic in some detail [6], and since nothing has been published in the interim that seriously modifies the conclusions in that overview it will not be examined in any depth here. In brief, a common theme in these processes is the capture of MT plus ends by sites at the leading edge of migrating cells. In the case of the EB1 interactions with CLIP-170 and the dynein/dynactin complex [61], the interaction between EB1 and APC [39, 62], and the interaction between EB1 and spectroplakins [16, 44], this attachment is likely to take the form of a linkage to cortical actin. Linkage mediated by EB1/CLASP interactions may also be dependent upon CLASP associations with proteins that directly bind to specific plasma membrane lipids [63], while an actin-independent interaction between the APC/EB1 complex and the cortex mediated by the PDZ domain protein Dlg1 has also been described [40]. Interactions between many of the components in these apparently discrete processes seems inevitable at the leading edge of a migrating cell, so defining whether cross-talk occurs and how the pathways might be integrated and regulated should keep investigators busy for some time to come.

## Roles for EB1 at kinetochores

The cortex of the cell is only one structure encountered by microtubule plus ends. Arguably the most important and certainly the most conserved EB protein interactions occur in the mitotic cell. EB proteins may contribute to spindle positioning [64–66] through MT cortical capture mechanisms analogous to those seen in migrating cells, and they have also been implicated in MT interactions with kinetochores (see [67] for a review of this subject). Interest in this topic has been driven by the possibility that loss of the interaction between EB1 and APC in colorectal cancer cells might contribute to tumour development and aneuploidy.

The dynein/dynactin complex, CLIP-170, LIS1 and CLASP1 all associate with kinetochores in the absence of MTs [68–70]. In contrast, APC is reported to only associate with kinetochores bound to MTs [71, 72], whereas EB1 is only present at kinetochores associated with growing MTs [73]. The function of +TIPs at the kinetochore has been extensively studied. CLIP-170 is involved in MT capture by this structure [74], while CLASP1 is thought to control the dynamics of captured MTs [75]. Suppressing the function of either protein causes activation of the spindle assembly checkpoint and mitotic arrest. Interference with dynein/dynactin function also prevents chromosomal congression and causes mitotic arrest [76, reviewed in 77]. Suppression of EB1 or APC expression does not trigger mitotic arrest but gives rise to defects in metaphase chromosomal alignment and anaphase segregation [65, 66, 72, 78], a phenotypic dif-

ference that suggests that any potential EB1 interactions with the 'resident' +TIPs at kinetochores may not play a major role in their function at this site. However, it remained unclear why misaligned chromosomes in cells lacking EB1 or APC did not induce mitotic arrest. Recent work has gone a long way to clarifying this issue [65, 66]. By depleting different kinetochore-associated MAPs in parallel and directly comparing the mitotic phenotypes observed, these studies confirmed that although loss of EB1 or APC did not affect chromosomal congression, it prevented the formation of a correctly ordered metaphase plate. Draviam et al. [66] went on to show that this drove a low but significant level of chromosomal missegregation during anaphase, an effect that could be replicated by the expression of a dominant-negative EB1 deletion mutant protein in transfected cells. Combined with the observation of Green et al. [65] suggesting that mutant truncated APC inhibits the interaction of the wild-type protein with EB1 in a dominant-negative manner, this work suggests that loss of the APC/EB1 interaction in mitotic cells causes defects that are not sensed by the spindle assembly checkpoint but are significant enough to drive sporadic chromosome loss and might therefore contribute to aneuploidy in cancers caused by APC mutation.

Even after these careful studies, many questions about the potential function of an APC/EB1 interaction in the mitotic cell remain to be addressed. For example, the APC interaction with EB1 is clearly sensitive to APC phosphorylation [38, 54], possibly mediated by CDK1 [38, 54, 79]. This would imply that APC/EB1 interactions are suppressed until anaphase unless APC phosphorylation could be locally suppressed during the earlier stages of mitosis. In addition, although a role for APC and EB1 in spindle positioning has been confirmed [64–66], its mechanistic basis in mammalian cells remains unclear.

## A role for +TIPs at MT minus ends

Perhaps because the localisation to growing MT plus ends is so striking, it is easy to overlook the fact that some +TIPs also exhibit other intracellular localisations. One of these is at the centrosome where, for example, EB1 [9, 10] and p150Glued [80] can be found. Interest in this localisation was initially limited since it appeared to represent a minor fraction of the population of the +TIPs concerned and because the centrosome is the organelle where new microtubules are nucleated. A +TIP presence at this structure would therefore seem to be inevitable, since they should bind to the end of nascent MTs. The first indication that +TIPs might have a specific role to play at the centrosome came from studies on p150Glued. Inhibiting p150Glued function in cells that normally exhibited a radial MT array revealed a relaxation in the focusing of MT minus ends at the centrosome [80, 81]. Work in our laboratory then characterised a direct interaction between p150Glued and EB1. We used the expression of dominant-negative EB1 protein fragments in an attempt to reveal a function for this interaction within cells. The most striking phenotype observed was a severe defect in MT anchorage at the centrosome, leading to the loss of the normal radial MT array [32].

Subsequently, a detailed analysis indicated that EB1 and its binding partner APC localised to the mother centriole within the centrosome independently of MTs. Knockdown of EB1 expression using siRNA inhibited MT anchorage at the centrosome, and it was suggested that APC might regulate the function of the EB1-p150Glued interaction at this site [82]. However, the most recent work on this topic suggests that the phenotypes observed in these studies may not solely result from loss of the EB1/p150Glued/APC interactions. Yan and colleagues [83] have identified an interaction between EB1 and the FGFR1 oncogene partner (FOP) protein. FOP interacts with another centrosomal protein, CAP350. CAP350 mediates the centrosomal localisation of FOP, which in turn appears to play a major role in the centrosomal localisation of EB1. Decreasing the expression any of these three proteins using siRNA inhibited MT anchorage at centrosomes. This study poses some interesting questions about the function of +TIPs at the centrosome and opens new avenues of investigation. For example, Yan et al. suggest that the CAP350/FOP complex provides centrosomal docking sites for EB1, which then capture freshly nucleated MTs in conjunction with p150Glued. As previously noted, the binding of EB1 ligands (except tubulin or MTs) has so far proven to be both mutually exclusive (i.e. [7, 32]) and likely to activate the MT growth-promoting activity of EB1 [48, 54]. If this is true for the EB1/FOP and EB1/p150Glued interactions, then the mechanistic basis of MT anchoring by these proteins should prove interesting. However, if the themes emerging from investigations of EB1 function at the plus end of the microtubule hold true elsewhere in the cell, we should expect any model of EB1 function at the centrosome to incorporate both an interaction with growing MT ends and EB1-dependent promotion of MT growth.

## +TIPS as experimental tools

Once the robust and reproducible nature of the association of +TIPs with growing MT ends was confirmed, it quickly became apparent that they could be used as tools with which to examine previously intractable aspects of MT growth and organisation. Prior to the commercial availability of reliable antibody reagents specific for proteins such as EB1, there was no easy way of examining a fixed cell and identifying which of the population of MT ends visible by tubulin immunostaining were growing

at the point of fixation. Furthermore, many cells (such as neurons) or cellular structures (such as the mitotic spindle) contain dense MT arrays that render the identification of individual MTs, let alone MT ends, impractical by conventional light or fluorescence microscopy. The ability to simply fix and immunostain for +TIPs has provided the opportunity to quantitatively examine at least one aspect of MT behaviour in fixed samples with high spatial resolution. The main drawback, of course, is that only growing tips are identified. Paused and shrinking tips can, at least in mammalian cells to date, only be identified by tubulin/+TIP coimmunostaining using the absence of +TIP staining as an indicator, while differentiating between paused and shrinking tips remains impossible. Further drawbacks are a) that MT growth is very sensitive to temperature and metabolic state, restricting the kind of samples that can be examined, and b) the fixation required to preserve the +TIP localisation at MT ends has to be both fast and capable of preserving good MT structure. In practical terms this means the use of cold organic solvents, typically methanol at –20 °C [9, 10], which is a comparatively poor fixative for preserving cellular morphology. In adherent cell types grown on coverslips these are not major problems, but they could prove to be obstacles if visualisation of growing MT ends in thick tissue samples or organotypic cultures is required.

Nevertheless, +TIP immunostaining has been used to quantitatively identify an increase in the length and number of astral MTs in mitotic mammalian cells [84] and has been used to identify polymerising ends in dense MT arrays such as those found in neuronal axons [85] and cultured myotubes [86]. Increasingly however, immunostaining is used in conjunction with the imaging of fluorescently tagged +TIPs expressed in transfected cells. Time-lapse imaging studies of MT behaviour within cells is a well-established technique, using either microinjected fluorescently tagged tubulin or the heterologous expression of GFP-tubulin. In most circumstances, both techniques share the same shortcomings as tubulin immunostaining. In addition, in living cells significant background interference from fluorescent tubulin not incorporated into MTs reduces imaging contrast, while the high density of MTs around the centrosome and increased sample thickness in the interior of the cell precludes the observation of MTs in this region. Most information about MT dynamics in vivo is therefore based upon observations made at the cell periphery where cytoplasmic background and MT densities are low. In comparison, +TIPs only label MT ends in living cells so MT density is less of a problem, while tip labelling is usually much brighter than any cytoplasmic background, allowing imaging in the cell interior or thick samples. Komarova and colleagues [87] exploited this by using GFP-CLIP-170 as a tool with which to study the growth of MTs from their nucleation at the centrosome. This revealed that most MT tips in the interior of the cell undergo persistent growth and that the growth/shrinkage cycle of 'classic' MT dynamics is only initiated when MT ends reach the cell periphery. The implications of this simple observation are profound, particularly when considering how cells might modulate the activity of specific MAPs to organise and modify MT networks.

MT tip tracking using fluorescently tagged +TIPs has since been applied in a wide variety of studies in almost every eukaryotic experimental system. In mammalian cells it has been used to examine MT organisation and dynamics during mitosis [73, 88, 89], to measure MT nucleation rates during centrosome maturation [90], to examine MT nucleation and dynamics in migrating wound-edge cells in monolayer culture [91], to describe MT rearrangements in response to plasma membrane disruption [92], to define MT organisation in fusing myotubes [93], to investigate MT growth in neurons (Fig. 2)

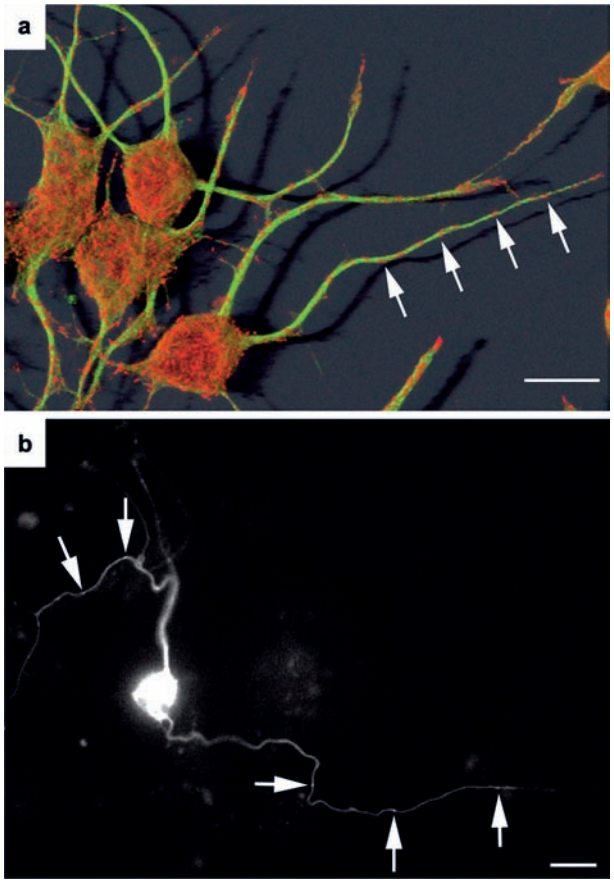


**Figure 2.** +TIP imaging reveals growing MT ends in dense MT arrays. (*a*) Methanol-fixed Neuro2A cells immunostained for MTs (green) and EB1 (red). Although individual MTs cannot be resolved in axons, individual EB1 comets are readily detectable (arrows), identifying MT plus ends growing at the point of fixation. Image produced from a stack of confocal sections using Imaris software. Bar, 10 µm. (*b*) Single frame from a time-lapse movie of a mouse embryonic cortical neuron in primary culture expressing EB1-GFP. Individual growing MT plus ends can be identified in neurites (arrows) and MT growth quantitatively analysed. Bar, 20 µm.

[85, 94, 95], to describe MT growth during protoplatelet formation by megakaryocytes [96] and to mark growing MT tips in a new protocol designed to examine MT dynamics in living cells [97]. All of these examples serve to illustrate the usefulness of +TIP imaging in basic studies of the microtubule cytoskeleton, though it should be noted that this approach has also been widely applied in non-mammalian systems as well. Biomedical scientists should also note the potential use of +TIP imaging in screening for, or defining the activity of, potential chemotherapeutic agents that might act through effects on MT dynamics. Experience in our laboratory suggests that of the +TIP-GFP fusion proteins currently available for use in time-lapse imaging in mammalian cells, EB1-GFP and EB3-GFP are probably the tools of choice on the basis of spatial resolution, lack of phenotype when expressed at low levels (although these fusions have a much reduced affinity for CAP-Gly containing +TIPs [33], so users should be aware of a potential for dominant-negative effects), lack of dependency upon $\alpha$-tubulin tyrosination status [30] and a 'clean' localisation to all growing MT ends. Of the two, EB3-GFP may turn out to be the best choice overall ([94]; J. M. Askham, unpublished observations).

## Future work

A number of studies have linked mammalian +TIPs to the function of molecular motors associated with the cytoskeleton, perhaps unsurprisingly in light of the role played by kinesins in targeting +TIPs to MT ends in yeast and fungi [98–100]. EB1 has been found to interact with the dynactin component p150Glued [32, 42] and the myosin V accessory protein melanophilin [21], while its ligand APC is known to exploit kinesin-dependent transport in its intracellular trafficking [19, 101]. Although recent work may have undermined the potential importance of +TIPs such as CLIP-170 and EB1 in intracellular membrane trafficking [59, 102] (though also see [14, 103]), it seems likely that progress in the investigation of +TIP-motor interactions in mammalian cells will be rapid. In *Drosophila* the minus end-directed kinesin ncd and the microtubule-depolymerising Kin I kinesin KLP10A have both been found to interact with EB1 [49, 104], as has the RhoGEF2 protein, a regulator of actomyosin-mediated contractility [105]. Intriguingly, the mammalian Kin I kinesin MCAK has recently been shown to track on growing MT ends [20]. This raises the possibility that factors that influence MT dynamics in opposing ways might modulate their competing activities via direct interactions. Defining how this is achieved, what the mechanisms that regulate these interactions are and how signal transduction pathways might locally influence these regulatory processes should ensure that interest in the actions and interactions of +TIPs continues to grow.

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