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Plus-End-Tracking Proteins and Their Interactions at Microtubule Ends

Review

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Microtubules are cytoskeletal elements that are essential for a large number of intracellular processes, including mitosis, cell differentiation and migration, and vesicle transport. In many cells, the microtubule network is organized in a radial manner, with one end of a microtubule (the minus end) embedded near the nucleus and the other end (the plus end) exploring cytoplasmic space, switching between episodes of growth and shrinkage. Mammalian plus-end-tracking proteins (+TIPs) localize to the ends of growing microtubules and regulate both the dynamic behavior of microtubules as well as the interactions of microtubules with other cellular components. Because of these crucial roles, +TIPs and the mechanisms underlying their association with microtubule ends have been intensively investigated. Results indicate that +TIPs reach microtubule ends by motor-mediated transport or diffusion. Individual +TIP molecules exchange rapidly on microtubule end-binding sites that are formed during microtubule polymerization and that have a slower turnover. Most +TIPs associate with the end-binding (EB) proteins, and appear to require these 'core' +TIPs for localization at microtubule ends. Accumulation of +TIPs may also involve structural features of the microtubule end and interactions with other +TIPs. This complexity makes it difficult to assign discrete roles to specific +TIPs. Given that +TIPs concentrate at microtubule ends and that each +TIP binds in a conformationally distinct manner, I propose that the ends of growing microtubules are 'nano-platforms' for productive interactions between selected proteins and that these interactions might persist and be functional elsewhere in the cytoplasm than at the microtubule end at which they originated.

Introduction

Microtubules are one of the three main types of cytoskeletal network in cells. They provide shape and structure to a cell and are also important for a large number of dynamic processes that take place inside cells. For example, microtubules form the mitotic spindle, a highly dynamic scaffold over which chromosomes are segregated during mitosis. Microtubules are also required for cell polarization and migration. Moreover, microtubules provide tracks for long-range intracellular transport, which takes place with the assistance of microtubule-based motor proteins. The enormous versatility in the use of the microtubule network requires careful regulation of its dynamic behavior and organization. This control is exerted at different levels: for example, by influencing the pool of free tubulin to change microtubule growth rate and, hence, catastrophe frequency; by severing microtubules; or by regulating microtubule dynamics through a large and heterogeneous group of proteins called microtubule-associated proteins, or MAPs. A subgroup of the MAPs — the plus-end-tracking proteins, or +TIPs [1] — specifically accumulates at the ends of growing microtubules. These +TIPs 'probe' the cytoplasm together with microtubules and are therefore in a strategic position to regulate the interactions between microtubules and other intracellular structures. Moreover, several +TIPs have been shown to control microtubule behavior. The structure and function of +TIPs in relation to microtubule structure and behavior and the mechanisms by which +TIPs accumulate at microtubule ends have been studied intensely. In this review, I describe recent progress in this area, focusing mainly on mammalian representatives of the +TIPs.

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Microtubule Structure and Behavior

Microtubules are assembled from α-tubulin–β-tubulin dimers, which interact in a head-to tail fashion to form linear protofilaments that in turn associate laterally, first into sheets and, subsequently, into hollow tubes with a diameter of about 25 nm (Figure 1). Most microtubules grown *in vivo* consist of 13 protofilaments. Two tubulin lattices have been proposed to exist — the A-type lattice where lateral bonds are formed between the α-tubulin of one protofilament and the β-tubulin of the adjacent protofilament, or the B-type lattice where lateral bonds are formed between two α-tubulins and two β-tubulins, each of adjacent protofilaments. In a B-type lattice (Figure 1) protofilaments are slightly staggered, resulting in a helical structure with a 'seam' where α- and β-tubulin subunits from adjacent protofilaments form lateral bonds. The distinct structure of this seam has been proposed to be a binding surface for MAPs, and this has been confirmed for the yeast +TIP Mal3p [2]. This protein is able to induce the formation of an A-type lattice *in vitro* [3], perhaps as a result of its preference for opposing α- and β-subunits on adjacent protofilaments. However, an A-type lattice does not appear to be present in mammalian cells [4]. Instead, a B-type lattice is found, indicating that the geometry of microtubules generated *in vivo* is determined by factors other than Mal3p or its mammalian homologues.

α-tubulin–β-tubulin dimers are polarized and, because they assemble in a head-to-tail fashion, the resulting tube is polarized as well, with a so-called plus end and minus end. In many cell types, the minus ends of microtubules are embedded in microtubule-organizing centers near the nucleus. Microtubule plus ends radiate into the cytoplasm and switch between episodes of growth and shrinkage, a process called dynamic instability [5]. The conversion of growth to shrinkage has been termed catastrophe, while conversion of shrinkage to growth is known as rescue. Dynamic instability allows the co-existence of fractions of growing, shrinking and stable (pausing) polymers and enables the cell to constantly probe the cytoplasm and to adapt quickly to a changing environment. Both microtubule polymerization and depolymerization can generate forces [6,7] that are comparable to forces generated by motor proteins and can be used to push vesicles or cell membranes, pull chromosomes apart during mitosis and stabilize the nucleus in the cell center.

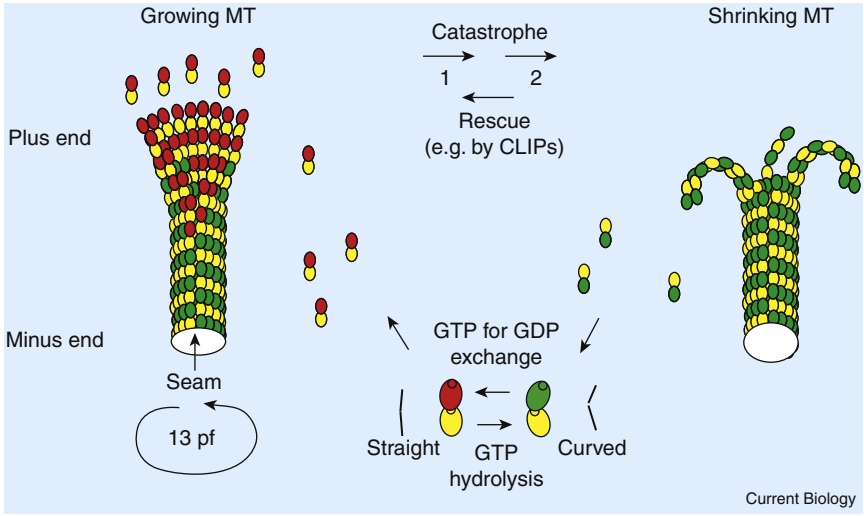

Figure 1. Microtubule structure and dynamics.

Microtubules (MT) are composed of 13 protofilaments of α-tubulin–β-tubulin heterodimers that exist as GTP-bound (red/yellow ellipses) or GDP-bound (green/yellow ellipses) forms. Growth and shrinkage of microtubules occurs mainly at the plus end. During polymerization, GTP-loaded tubulin subunits are assembled head-to-tail into protofilaments, which might first form sheets and then fold up into a tube. Here, the growing microtubule plus end is depicted as transitioning from a sheet into a hollow tube. Microtubules with a B-type lattice and 13 protofilaments (13 pf) have a seam, a discontinuity in the surface of the microtubule wall (its position is indicated with an arrow), generated by the helical pitch in adjacent protofilaments. GTP hydrolysis occurs shortly after incorporation (conversion of red to green ellipses). Different events (indicated by numbered arrows) lead to catastrophe and microtubule shrinkage. A shrinking microtubule is characterized by curved protofilaments, which is caused by the preference of GDP-bound tubulin to adopt a curved conformation. Microtubules can undergo rescue, for example due to the action of the CLIPs. The polymerization–depolymerization cycle is completed by the exchange of GDP with GTP (lower part of image).

Free tubulin dimers bind two GTP molecules — one at the non-exchangeable N-site of α-tubulin and one at the exchangeable E-site of β-tubulin. Incorporation of tubulin into growing microtubules results in interactions between consecutively incorporated tubulin dimers that are thought to trigger hydrolysis of GTP in the E-site. Therefore, β-tubulin along the length of a microtubule is bound to GDP, while the plus end is capped by a layer of GTP-bound β-tubulin [8] (Figure 1). Using an antibody that recognizes GTP-bound tubulin, it was recently shown that small discontinuities are present inside the microtubule lattice, indicating that not all β-tubulin subunits in microtubules are in the same conformational state [9]. GTP-bound tubulin is relatively straight and allows microtubule polymerization, while GDP-bound tubulin favors a more curved conformation. Lateral and longitudinal bonds between adjacent tubulin dimers prevent the GDP-bound tubulin in the microtubule lattice from adopting the curved conformation that is energetically favorable for free GDP–tubulin. To prevent microtubule depolymerization by the 'peeling off' of protofilaments (Figure 1), the final layer of GDP-bound tubulin at the plus end of the microtubule needs to be protected by a terminal layer of GTP-bound tubulin subunits that forms strong lateral bonds. This 'GTP cap' has been the focus of many studies, but no consensus has been reached about the size and precise function of this cap. While earlier work suggested that a single layer of GTP-bound tubulin might be sufficient to stabilize microtubules, more recent studies indicate that individual protofilaments contain a variable number of GTP-bound tubulin molecules at the plus end, and that individual protofilaments can even shorten when the microtubule as a whole is in a growth phase [10]. Once the cap is removed the preference of GDP-bound tubulin subunits to adopt a curved conformation causes the breaking of lateral bonds and results in microtubule catastrophe. *In vitro* experiments have shown an inverse correlation between microtubule growth rate and catastrophe frequency [11]. However, the speed of tubulin incorporation might be highly variable at the molecular level, and it is unclear whether tubulin oligomers are incorporated at microtubule ends [10,12]. Interestingly, microtubule catastrophe is not a single-step process, but appears to involve more than one event [13]. Depending on the nature of the microtubule cap, different events might be envisioned: for example, closure of the tubulin sheet up to the very end of the microtubule, or a reduction in the rate of microtubule assembly, allowing GTP hydrolysis to catch up with GTP–tubulin polymerization and expose GDP–tubulin at the microtubule end.

Role of +TIPs in Regulating Microtubule Behavior

Dynamic plus-end-tracking was first described for a GFP-labeled form of CLIP-170; in time-lapse imaging experiments this fusion protein was visible as "cellular fireworks" of "comet-like" fluorescent dashes moving mostly from the cell center to the periphery [14]. Using specific antibodies and appropriate fixation conditions, fluorescent comets can be visualized in immunofluorescence images of fixed cells (see Figure 2 for an example of a combined analysis of the localization of the end-binding (EB) protein EB1 and CLIPs). The family of +TIPs has been growing steadily and, to date, more than 20 +TIPs have been described (for an overview, see [15]).

+TIPs influence microtubule dynamic instability [15] and are involved in microtubule nucleation [16,17]. A role for multiple +TIPs has also been established at the leading edge of the cell. In motile fibroblasts, for example, a subset of microtubules is oriented towards the leading edge and becomes selectively stabilized [18]. Specific signaling pathways that are activated at the leading edge of the cell mobilize downstream effectors to recruit stable, leading-edge-oriented microtubules. A number of signaling molecules involved in these pathways have been identified: for example, lysophosphatidic acid (LPA), which triggers a pathway dependent on the Rho GTPase and the formin mDia [19]; and phosphatidylinositol 3-kinase, acting via glycogen synthase kinase 3 (GSK3) [20]. Several +TIPs have been implicated in selective microtubule stabilization, including adenomatous polyposis coli (APC) [21], CLIP-170-associated proteins (CLASPs) [20], the spectraplakin ACF7 [22], and CLIP-170 [23]. Interestingly, in addition to binding

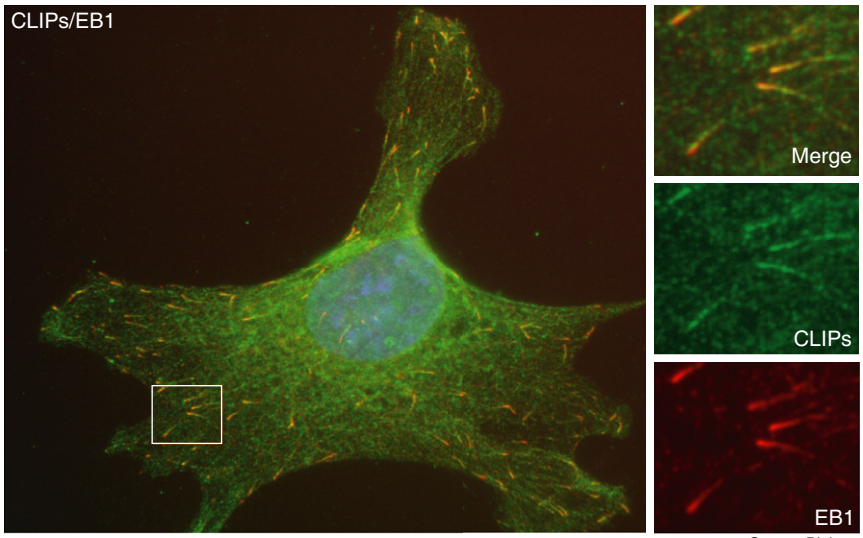

Figure 2. Intracellular distribution of +TIPs.
Image of a cultured fibroblast, fixed and stained afterwards for endogenous EB1 (red) and CLIPs (green). The nucleus is outlined in blue. Note the distribution of the two +TIPs at the distal ends of microtubules (image kindly provided by Jeffrey van Haren).

microtubule ends, these +TIPs are, directly or indirectly, connected to the actin network. Furthermore, +TIPs may influence each other's activity: for example, both APC [24] and CLIP-170 [25] have been shown to bind mDia1; ACF7 influences and binds CLASPs [26,27]; both CLASPs and APC are regulated by GSK3 [21,28,29]; and CLIP-170, APC and CLASPs all interact with IQGAP1 [23,29,30], an integrator of cytoskeletal function. These data suggest that the leading edge of the cell contains a complex and interconnected network of +TIPs and other proteins involved in signal transduction and microtubule stabilization. CLIP-170 has recently also been shown to be involved in the capture of *Xenopus* melanophores for minus-end-directed transport along microtubules [31]. +TIPs therefore link microtubules to intracellular structures, including not only vesicles and the cell membrane, but also focal adhesions, the actin and intermediate filament cytoskeletons, and kinetochores.

More than 20 years ago, a 'search and capture' model was proposed [32], in which dynamic microtubules, exploring cytoplasmic space, are captured at specific cellular sites and are transiently stabilized to allow, for example, the directed transport of vesicles from and towards the plasma membrane, or the separation of chromosomes to daughter cells. The discovery of the +TIPs as essential linking factors between microtubules and intracellular structures validated this model at the molecular level, although it should be noted that the 'comet'-like dash caused by the binding of a +TIP to microtubule ends is often larger than 1 μm, allowing microtubules and +TIPs to undergo lateral as well as end-on interactions. Lateral interactions are more likely to occur and may actually be more important than end-on interactions between microtubules and +TIPs.

Classification of +TIPs

Although +TIPs have a common ability to track growing microtubule plus ends *in vivo*, they do not have a common microtubule-end-binding domain and the molecular mechanisms by which +TIPs recognize microtubule ends differ. Given the availability of protein sequences and several crystal structures, +TIPs can be classified according to sequence homologies and the domains and/or mechanisms involved in microtubule-end tracking (Table 1). It should be noted that the classification below does not cover all +TIPs: for example, the protein LIS1, which is important for neuronal migration and brain function [33], is a known +TIP [34], but the domain involved in this activity has not been precisely delineated (Table 1).

End-Binding (EB) proteins

There are three mammalian end binding (EB), or EB1-like, proteins: EB1, EB2 and EB3 [35]. EB1 was first described as a protein that interacts with the carboxyl terminus of APC [36], hence the name 'end-binding'. EB proteins are relatively small dimers, with each monomer containing two conserved domains connected by a linker region. The amino-terminal calponin homology (CH) domain mediates the interaction with microtubules and is sufficient for the recognition of growing microtubule ends [37,38]. A coiled-coil domain is present at the carboxyl terminus of EB proteins and is responsible for homo- and heterodimerization [39]: this domain contains a unique motif called the EB homology (EBH) domain, which mediates the interaction of EB1-like proteins with other +TIPs [40]. The carboxy-terminal tail (~25 residues) of EB proteins is flexible and the ultimate carboxy-terminal amino acids — the EEY/F-motif — are homologous to the carboxyl terminus of tyrosinated α-tubulins and CLIP-170 [41,42]. The crystal structures of both the CH and coiled-coil domains of EB1-like proteins have been solved (see [43] for an overview) and have led to the hypothesis that the CH domain binds microtubules primarily via electrostatic and hydrophobic interactions. The coiled-coil domain of EB proteins ends with a four-helix bundle and contains a hydrophobic cavity and a polar rim. The coiled-coil domain and carboxyl terminus of the EB proteins binds to SxIP and CAP-Gly motifs, which are found in other +TIPs and discussed in more detail in the following subsections. The structural organization of EB proteins allows for their autoinhibition in the cytoplasm [44], and for interactions with microtubules and other +TIPs at plus ends.

Regarding the function of the EB proteins, there are interesting apparent discrepancies between *in vitro* and *in vivo* experiments. For example, in two *in vitro* studies the addition of EB proteins leads to higher microtubule growth rates as well as higher catastrophe frequencies [38,45]. Although these studies differ in other effects on microtubule dynamics, these data indicate that EB binding to *in vitro* grown microtubules changes the structure of the microtubule end, thereby inducing catastrophe. In contrast to the *in vitro* studies, depletion of EB1 in mouse fibroblasts promotes microtubule pausing and reduces the time microtubules spend growing [46], while knockdown studies in CHO cells reveal a role for EB proteins in suppressing catastrophes instead of enhancing them [38]. One explanation for these different results from

in vitro and *in vivo* studies is that depletion of EB proteins in cells affects both their intrinsic activity as well as their association with other microtubule-regulating factors, whereas *in vitro* studies with purified components examine the intrinsic activity of EB proteins. Furthermore, the effects of an EB knockdown may vary depending on the dominant microtubule-regulatory activity present in a given cell type [38,47]. Finally, it is noteworthy that the *in vitro* effects of Mal3p, the fission yeast EB1 homolog, on yeast microtubules appears to be different from that of Mal3p and EB1 on mammalian microtubules: in the yeast system Mal3p masks catastrophe events [48] and induces formation of an A-type microtubule lattice [3].

+TIPs with a CAP-Gly Domain

As mentioned above, CLIP-170 was the first +TIP identified [14]. CLIP-170 contains so-called CAP-Gly domains [49], through which it binds to microtubules and microtubule ends. These domains are located at the amino terminus and are linked by a long coiled-coil region to two zinc knuckles in the carboxyl terminus [50]. The coiled-coil region forms a rod-shaped dimerization domain, but it also contains kinks [51] at which the rod might bend to allow intramolecular interactions of the amino-terminal CAP-Gly domains with the carboxy-terminal zinc knuckles [52] and EEY/F-motif [41,53] (see below). The closest homolog of CLIP-170 in vertebrates is CLIP-115, which is mainly expressed in the brain, lacks the carboxy-terminal zinc knuckles [54] and localizes to growing microtubule plus ends [55]. Another CAP-Gly-domain-containing protein is p150^{glued}, which is part of the dynactin complex, a large regulatory module of microtubule-based motor proteins [56].

The crystal structures of several CAP-Gly domains have been solved, either alone or in association with other protein domains or peptides (for an overview, see [43]). The structure of the carboxyl terminus of EB1 together with the CAP-Gly domain of p150^{glued} revealed that CAP-Gly domains are EEY/F recognition modules [41]. Furthermore, CAP-Gly domains bind to the conserved EBH domain. As previously mentioned, the acidic–aromatic EEY/F motif is present at the carboxyl terminus of EB1-like proteins, α-tubulin, and CLIP-170. In α-tubulin, the carboxy-terminal tyrosine can be removed by a tubulin tyrosine carboxypeptidase (TTCP) and added back by tubulin-tyrosine ligase (TTL). TTL has been studied extensively [57,58], but TTCP has not been identified yet. TTCP and TTL differ in their substrate preference: TTCP preferentially detyrosinates the carboxyl termini of α-tubulin in microtubules, while TTL tyrosinates free tubulin dimers. As a consequence, microtubules acquire detyrosinated α-tubulin over time. CAP-Gly-domain-containing +TIPs do not bind the ends of growing microtubules in TTL-deficient cells [58], indicating that the carboxy-terminal tyrosine of α-tubulin is a major determinant of the accumulation of CAP-Gly-domain-containing proteins at microtubule plus ends. The EEY/F motif at the carboxyl terminus of CLIP-170 allows for an intramolecular CAP-Gly–EEY/F interaction and explains the autoinhibition of this +TIP [59].

Whereas Tip1p, the CLIP-170 homolog in fission yeast, is an anti-catastrophe factor [60], mammalian CLIPs were shown to promote microtubule rescues in CHO cells [61] and *in vitro* [62]. This raises an interesting question: how is it possible that CLIPs associate with the ends of growing microtubules yet function at the ends of shrinking microtubules? It has been suggested that discontinuities in the microtubule lattice, possibly containing GTP-bound tubulin, serve as preferred sites of microtubule rescue and that the CLIPs act as the rescue factors [9]. However, if the latter assumption were true, then one would also expect to see a role for mammalian CLIPs in regulating microtubule dynamics at growing microtubule ends, but this has not been documented. Nevertheless, it can be concluded that CLIPs are positive regulators of microtubule growth.

Table 1. Classification of +TIPs.

Classification	+TIP*	Homologs†	Interaction with other +TIPs
'Core' +TIPs	EB1-like proteins	Bim1 (*Sc*) Mal3 (*Sp*)	Most known +TIPs
CAP-Gly domain	CLIP-170	CLIP-190 (*Dm*) Bik1 (*Sc*) Tip1 (*Sp*)	EB1, CLIP-170, CLIP-115, p150^{glued}, CLASP1,2, MCAK, LIS1
	CLIP-115	–	EB1, CLIP-170, CLASP1,2
	p150^{glued}	NudM (*An*) Ssm4 (*Sp*)	EB1, CLIP-170
SxIP motif	CLASP1,2	Orbit/Mast (*Dm*) Stu1 (*Sc*) Peg1 (*Sp*)	EB1, CLIP-170, CLIP-115, ACF7
	APC	Kar9p (*Sc*)	EB1, MCAK
	ACF7	Shot/Kakapo (*Dm*)	EB1, CLASP1,2
	STIM1	–	EB1
	MCAK	Klp10A (*Dm*) XKCM1 (*Xl*)	EB1, CLIP-170, APC, Tip150
	Tip150	–	EB1, MCAK
	Navigators	–	Unknown
	Melanophilin	–	EB1
	p140Cap	–	EB1
	CDK5RAP2	–	EB1
	RhoGEF2 (*Dm*)	–	EB1
	DDA3	–	EB1
TOG domain	Ch-TOG	Msps (*Dm*) XMAP215 (*Xl*) Stu2 (*Sc*)	EB1
Not classified	LIS1 NudA (*An*)	NudF (*An*) Dynein heavy chain	CLIP-170, p150^{glued} p150^{glued}, LIS1

*Mammalian +TIPs are shown, otherwise the species is indicated: *Aspergillus nidulans* (*An*), *Drosophila melanogaster* (*Dm*), *Saccharomyces cerevisiae* (*Sc*), *Schizosaccharomyces pombe* (*Sp*), *Xenopus laevis* (*Xl*).
†Names of homologs are shown if they are different.

+TIPs with a SxIP Motif

Several +TIPs, including the CLASPs, APC, microtubule-actin crosslinking factor (MACF), the calcium sensor STIM1, and mitotic centromere-associated kinesin (MCAK), do not appear to use a defined and structured domain for targeting to microtubule ends. Crystallization studies revealed that a conserved Ile-Pro (IP) dipeptide in APC and MACF is critical for interaction with EB1 [63]. The EB1-binding region in CLASPs also contains IP dipeptides, which are embedded in a stretch of sequence similarity between CLASPs, APC, and MACF [59]. Recently, it was demonstrated that a short Ser–x–Ile–Pro (SxIP) polypeptide motif is actually used by many +TIPs for binding to the EBH domain and that this small 'signature' is also required for the EB1-dependent localization of these +TIPs to microtubule ends [40]. The SxIP motif has therefore been termed a general microtubule tip localization signal (MtLS). Given that the SxIP motif is small, it is likely to occur in many more proteins than the +TIPs identified to date (listed in Table 1), therefore raising the interesting issue

of what prevents such proteins from tracking microtubule ends under normal conditions. Since both the SxIP motif and certain basic residues surrounding this motif appear to be important for EB1 binding and microtubule-end tracking [40], this dual requirement might limit the number of proteins that are able to bind microtubule ends. Nevertheless, the +TIP family might be larger than anticipated.

CLASP2 contains two adjacent SxIP motifs [59], which increases the affinity of this +TIP for growing microtubule plus ends [40]. CLASP2 is monomeric [27] and one CLASP2 molecule is therefore able to bind to the two EBH domains in an EB dimer. Interestingly, the EB1–SxIP interaction is negatively regulated by phosphorylation [40]. In agreement with this, serine residues near the SxIP motifs of CLASP2 are phosphorylated by GSK3β, which impairs microtubule plus-end tracking by CLASP2 [28,29].

A special SxIP-motif-containing protein is MCAK. This protein belongs to the kinesin family, which has been divided into 14 subfamilies [64]. Kinesins can be grouped according to the position of their motor domain. The microtubule destabilizing Kin I kinesins of the kinesin-13 subfamily contain an internal catalytic domain and do not move cargo along microtubules, but use the energy from ATP hydrolysis to induce a bent conformation of protofilaments that facilitates depolymerization [65]. MCAK is a prominent member of this kinesin-13 family. *In vitro* studies revealed that MCAK reaches microtubule ends by diffusion over the microtubule lattice [66]; however, it is able to track the ends of growing microtubules in cells [67]. It is unclear whether, or how, the microtubule-depolymerizing activity of MCAK is regulated by EB1. Intriguingly, MCAK activity is negatively regulated by detyrosinated microtubules [68].

+TIPs with a TOG Domain

XMAP215 is the founding member of the XMAP215/Dis1/Tog protein family, which is conserved from yeast to humans [69]. Members of the XMAP215 family contain several TOG domains, crystal structures of which have been solved [70,71]. Free XMAP215 is a thin rod with multiple flexible regions, whereas XMAP215 bound to tubulin forms a well-ordered globular complex, 'embracing' a tubulin dimer [72]. XMAP215 increases microtubule growth rate ~10-fold [73], indicating it is involved in accelerating microtubule growth. Consistent with this observation, interfering with Dis1/XMAP215 function decreases microtubule growth rates and depletion of Dis1/XMAP215/Tog proteins leads to shorter spindles and defects in spindle morphology [74,75]. It has been suggested that XMAP215 speeds up microtubule growth by facilitating the incorporation of tubulin oligomers [12]. However, recent data show that XMAP215 acts as a processive tubulin polymerase, which, while remaining bound to microtubule ends, supports multiple rounds of addition of individual tubulin dimers [72]. Furthermore, XMAP215 is able to antagonize the activity of the catastrophe-promoting kinesin, MCAK [47,75]. Interestingly, CLASPs contain a single TOG domain and several TOG-like domains, indicating a functional overlap with XMAP215/Dis1/Tog proteins [71].

Accumulation of +TIPs at Microtubule Ends

Since the first description of CLIP-170 as a +TIP, the mechanisms that lead to microtubule plus-end accumulation have been studied intensely, and several theories have been proposed. I will first describe mechanisms that deliver +TIPs to microtubule ends and then focus on features of the microtubule end that might be recognized by the +TIPs.

Cytoplasmic Diffusion and Fast Exchange at Microtubule Ends

Initially, when GFP–CLIP-170 was seen to 'move' in fluorescent comets from the nucleus to the cell periphery, a 'treadmilling' mechanism underlying this movement was envisioned — i.e. CLIP-170 binds to the polymerizing microtubule end and dissociates after 1–3 seconds [14]. In this view the fluorescent comet-like distribution of +TIPs at microtubule ends is linked to association/dissociation of +TIPs. Dissociation could be triggered either by conformational changes of the microtubule, or by modifications and/or conformational changes of the +TIP itself. Alternatively, it was proposed that CLIP-170 and other +TIPs localize to the plus-end through co-polymerization with tubulin. Co-polymerization requires that +TIPs have affinity for free tubulin dimers.

Using inverted fluorescence recovery after photobleaching (iFRAP) it was shown that Tip1p and Mal3p, the fission yeast homologs of CLIP-170 and EB1, are retained at microtubule ends with different time frames [76], indicative of different mechanisms of protein turnover at microtubule ends. iFRAP only measures loss of fluorescence, so it is not clear whether individual molecules rebind microtubule ends or dissociate after one round of binding. Single-molecule experiments on *in vitro* growing microtubule ends [77,78] as well as fast FRAP measurements in living cells [79] subsequently revealed that CLIP-170 and EB proteins rapidly exchange on microtubule ends. Increasing the tubulin concentration in the *in vitro* studies resulted in increased microtubule growth rates but this was not accompanied by increased EB protein fluorescence at the microtubule end. When considered together, these data argue against the original treadmilling and co-polymerization concepts as mechanisms underlying the turnover of +TIPs at microtubule ends. Instead, a fast exchange model for the binding and release of +TIPs can be proposed (Figure 3). In this view, microtubule polymerization generates a vast number of binding sites that disappear exponentially; each site can bind and release +TIP molecules several times before disappearing [78,79]. Diffusion of +TIPs appears to be rate-limiting for the binding on microtubule plus ends and CLIP-170 binds microtubule ends with relatively low affinity (~0.44 μM). It is important to realize that, although a comet moves through the cytoplasm, individual +TIP molecules do not: they are actually transiently immobilized at the ends of growing microtubules (the acronym +TIP can also be read as 'transient immobilization of proteins at + ends').

Motor-Driven Transport, 1D Diffusion and Hitchhiking

Kinesin-mediated motor-driven transport of +TIPs towards the microtubule plus end may also result in accumulation of a protein at microtubule ends. However, this occurs only if kinesin transport is faster than microtubule growth and if the +TIP is somehow retained when it reaches the plus end, i.e. the kinesin does not just move along the microtubule and fall off at the plus end taking the +TIP with it. Motor-driven transport was shown for the yeast homologs of CLIP-170, Bik1p in budding yeast, and Tip1p in fission yeast [76,80], which both depend on a kinesin (Kip2p in budding yeast and Tea2p in fission yeast) for their accumulation at the plus end. Motor-driven transport of Tip1p and Tea2p

could be recapitulated *in vitro* and was shown to depend on Mal3p [78]. APC in mammalian cells can also be transported towards plus ends by kinesin [81]. Thus, motor-driven transport is an established means of delivering +TIPs in different species.

Decreasing the dimensions in which a molecule can diffuse decreases the time it takes for this molecule to reach a specific target. This 'reduction in dimensionality' was first proposed for proteins that bind to a specific DNA target sequence. A similar mechanism might facilitate the accumulation of proteins at microtubule plus-ends. One-dimensional diffusion along the microtubule lattice has been proposed for MCAK [66] and XMAP215 [72], which is interesting because these two proteins are major opposing microtubule regulatory factors [47,75]. Furthermore, a basic microtubule-binding domain of $p150^{glued}$ was also shown to 'skate' along microtubules [82]. It is noteworthy that the residence time of +TIPs that diffuse along a microtubule is relatively long compared with that of +TIPs that rapidly exchange at the microtubule ends.

Hitchhiking was defined as plus-end delivery mediated through binding to another +TIP [83]. To date, the EB proteins are considered to be the conserved core components of microtubule ends, because they associate autonomously with growing microtubule ends *in vitro* [77,78,84,85], yet interact with most other +TIPs [86]. Hitchhiking on EB proteins therefore appears to be a general property of +TIPs. In fact, the only +TIPs that have been shown to track microtubule ends autonomously are the EB proteins themselves and XMAP215. However, EB1 and XMAP215 do interact [47,87] and this association may serve to prevent EB1 binding to the microtubule destabilizer MCAK. Interestingly, besides EB1, MCAK appears to require yet another +TIP, Tip150, to efficiently localize at microtubule ends [88]. Tip150, in turn, is dependent on EB1 for its plus-end accumulation. Thus, MCAK may reach microtubule ends by diffusion, but might be optimally retained at the end by the combinatorial action of EB1 and Tip150. The example of MCAK supports the notion that +TIPs use different modes for delivery to, and retention at, microtubule ends. The same holds true for APC, which can accumulate in EB-dependent and -independent manners [81,89]. Moreover, delivery mechanisms may vary between orthologs of the same +TIP. CLIP-170, for example, is proposed to rapidly exchange on mammalian microtubules [79] but be delivered to microtubule ends by motor proteins in yeast [76,80].

Microtubule-End Recognition and the Distribution of Microtubule-End Binding Sites

The fast-exchange model implies that the fluorescent comet seen along a microtubule end correlates with the disappearance of binding sites for +TIPs. The comet tail length (expressed as the length at which half the fluorescence has disappeared; Figure 4) reflects the distribution of +TIP-binding sites along the microtubule, whereas the decoration time measured inside a region of interest near the microtubule end (expressed as the time it takes for half the fluorescence in this particular region to disappear; Figure 4) reflects the lifetime of the binding sites.

If we acknowledge that the localization of +TIP fluorescence at microtubule ends reflects the distribution of +TIP binding sites, then what features might constitute these sites? It has been suggested that the core EB proteins might recognize the GTP cap [90] and/or tubulin sheets [45].

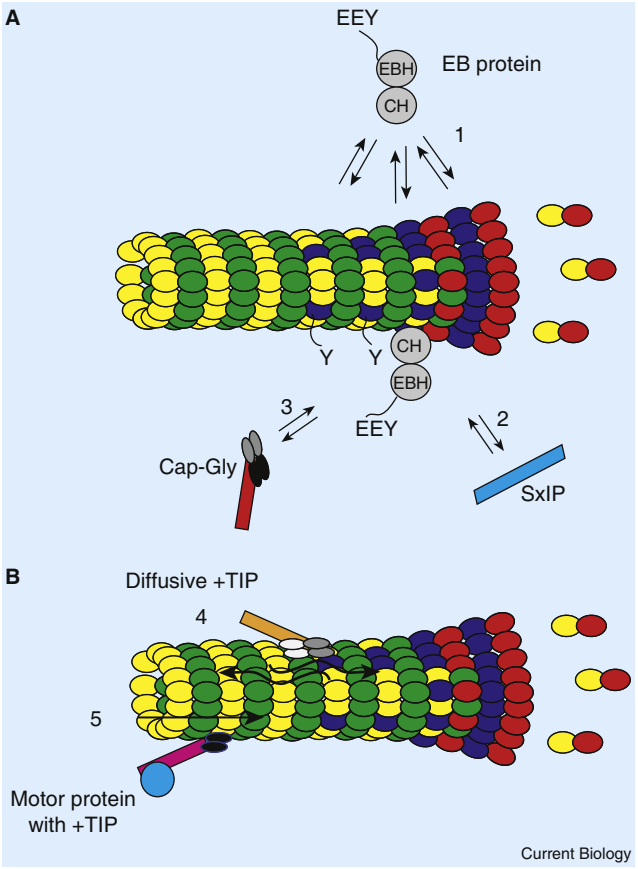

Figure 3. Delivery of +TIPs to microtubule ends.

(A) Diffusion and fast exchange. +TIPs reach microtubule ends by diffusion from the cytoplasm. The EB proteins bind microtubule ends autonomously using their calponin homology (CH) domain (1). Other +TIPs bind microtubule ends by hitchhiking (2), i.e. non-autonomously. For example, microtubule-end binding of a SxIP-motif protein is mediated by binding to the EB homology domain of the 'core' EB proteins. +TIPs may also accumulate using composite binding sites (3). For example, CAP-Gly-motif proteins recognize composite binding sites consisting of the carboxyl termini of EB proteins (curved lines with EEY) and tyrosinated α-tubulin (curved lines with Y). +TIPs exchange rapidly on individual binding sites (indicated in blue), while the ensemble of binding sites decays more slowly over time. (B) Lattice diffusion and motor-mediated transport. In the examples in panel (A), +TIPs reach microtubule ends by diffusion from the cytoplasm. One-dimensional diffusion along the microtubule lattice towards the microtubule end (4) is also possible. Finally, motor-mediated transport of +TIPs by kinesins (5) has also been shown to occur. An interesting possibility that has not been examined in detail is a combination of hitchhiking and diffusion along the lattice, away from the plus end.

However, EM studies indicate that the maximal sheet length of interphase microtubules in fibroblasts is 320 nm [91], whereas EB1 fluorescent comets can be over 1 μm long [42]. Thus, EB proteins might recognize features of microtubule ends in addition to GTP-bound tubulin and/or protofilament sheets. The EB proteins, in turn, are thought to provide essential (albeit partial) binding sites for virtually all other +TIPs. For example, *in vitro* plus-end tracking experiments show that CLIP-170 requires EB1 for association with microtubule ends [77,85], consistent with structural data. In addition, microtubule-end association of CLIP-170 also depends on the carboxy-terminal tyrosine of α-tubulin. This requirement might be more dominant *in vivo*, as CLIP-170 fails to

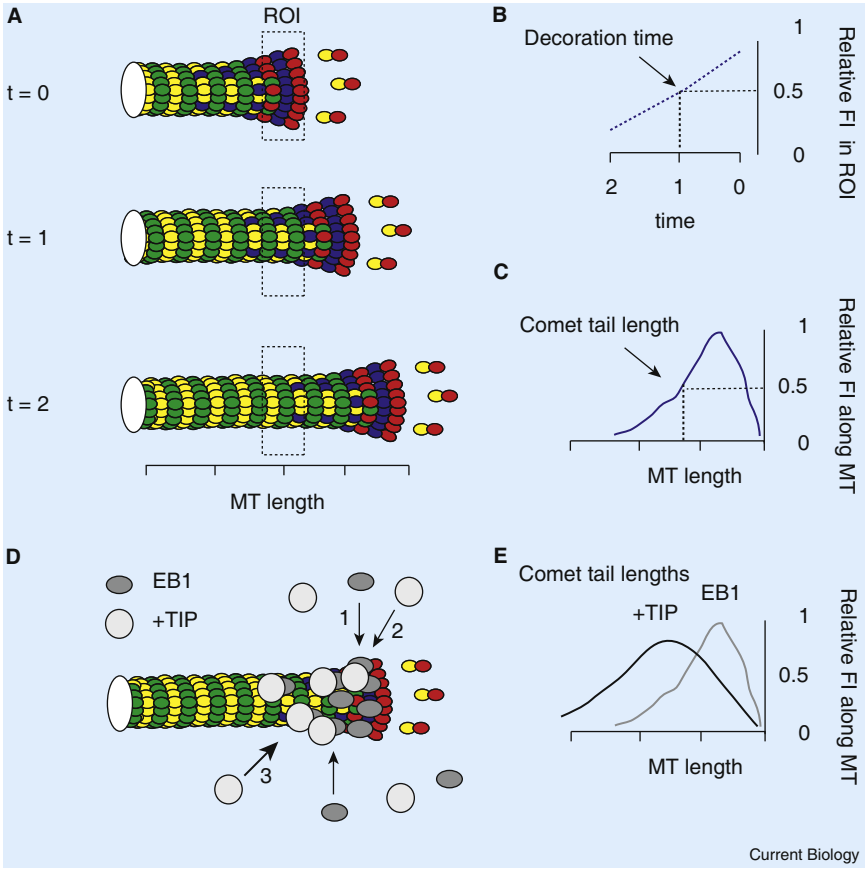

Figure 4. Distribution of microtubule end binding sites and +TIPs.

(A) Time course of growing microtubules (MT). A single microtubule is depicted at three time points. GTP-bound α-tubulin–β-tubulin heterodimers are depicted as red/yellow ellipses. The dynamic behavior of a fluorescently labeled +TIP, which correlates with the distribution of the +TIP-binding sites (depicted in blue), can be analyzed along the length of the microtubule, or over a period of time within a region of interest (ROI). (B) Decoration time. A fluorescent comet, which reveals the distribution of a fluorescently labeled +TIP on microtubule ends, disappears over a period of time inside a ROI near the microtubule end, due to the disappearance of binding sites. The decoration time is expressed as the time it takes for half the fluorescence (FI) to disappear, and reflects the lifetime of binding sites for the particular +TIP. (C) Comet tail length. A fluorescent comet tail seen along a microtubule end correlates with the disappearance of binding sites for a particular +TIP on the microtubule. The comet tail length is expressed as the length at which half the fluorescence has disappeared and reflects the distribution of +TIP-binding sites along the microtubule. (D) Model explaining the dissimilar distribution of EB1 and other +TIPs at microtubule ends. EB1 binds microtubule ends autonomously (1). EB1 crowding at the very end of a growing microtubule may mask binding sites for another +TIP on the microtubule surface, meaning that the +TIP accumulates through low-affinity hitchhiking on EB1 (2). As EB1 density decreases, the +TIP can bind composite sites on the microtubule end (3). See also Figure 3A. (E) Comet tail lengths of EB1 and another +TIP. The distribution of EB1 at microtubule ends follows that of its binding sites, with the highest concentration for the protein at the very proximal end followed by an exponential decline along the microtubule. For other +TIPs, the initial conversion of low (2) to higher (3) affinity binding, followed by the disappearance of binding sites, results in a trailing comet that reaches its maximum at a position distal to the maximum of EB1.

bind microtubule ends in cells that lack tyrosinated α-tubulin [12] but is recruited to microtubule ends in cells expressing a mutant form of EB1 that lacks the EEY domain [42]. Thus, CLIP-170 appears to use composite microtubule-end binding sites that may differ in their relative contribution to CLIP-170 binding.

Fluorescence imaging studies suggest that, for several +TIPs, the distribution of binding sites at microtubule ends does not exactly correlate with that of EB1, given that EB1 is more abundant at the very proximal tip of the microtubule whereas the other +TIPs are localized more distally (e.g. [92–94]). One explanation for this phenomenon is that EB1 crowding at the very end of a growing microtubule masks potential binding sites for other +TIPs on the microtubule surface (Figure 4D,E). These +TIPs can only accumulate at the microtubule end through low-affinity hitchhiking on EB1. As the number of binding sites for EB1 decay, EB1 density on the microtubule end decreases. At this point, binding sites on the microtubule surface become available for the +TIP, leading to a higher affinity interaction with EB1 and the microtubule surface, and a higher concentration of the +TIP at the microtubule end (Figure 4E). Conversely, the composite binding of a +TIP to EB1 and the microtubule surface might result in a prolonged EB1 dwell time on the microtubule end. In conclusion, there might be more than one end recognition mode for a particular +TIP on the same microtubule; this applies to EB proteins as well as other +TIPs, even though it was not revealed by fast FRAP studies [79]. Recent data reveal that CLIP-170 localization to a subset of microtubule ends in phagocytic cups is actually independent of EB1 [25], indicating that distinct end recognition modes may exist on different microtubules in the same area of the cell.

Post-translational modifications (e.g. phosphorylation) of +TIPs affect their interaction with EB proteins [40] and could also induce conformational changes in +TIPs, thereby masking, or revealing, additional microtubule binding surfaces on +TIPs. For example, the inactivation of GSK3, which normally phosphorylates CLASP2, leads to an enhanced interaction of CLASP2 with distal microtubule segments [20]. Thus, post-translational modifications can influence the binding behavior of +TIPs, leading to shorter, or longer, dwell times of the +TIP at microtubule ends, which in turn could lead to shorter, or longer, comets, and hence to altered microtubule behavior.

What other mechanisms could regulate the lifetime of microtubule-end binding sites of a particular +TIP and, possibly, microtubule dynamics? *In vitro* tracking studies using fluorescently labeled Mal3p showed that an increased microtubule growth rate due to higher tubulin concentration resulted in longer comet tail lengths (i.e. more binding sites along the length of a microtubule) but not different decoration

times [78]. Thus, *in vitro* the lifetime of Mal3p microtubule-end binding sites is not influenced by increased microtubule growth rates. Increasing EB1 concentration from 100 nM to 500 nM also resulted in an increased comet length *in vitro* [85]. This is a surprising result since addition of EB proteins *in vitro* is thought to increase catastrophe frequency by altering microtubule end structure [38,45]. Hence, one would expect that addition of EB proteins accelerates binding site turnover (i.e. reduces comet tail length). As discussed above, one explanation is that EB proteins recognize more than one type of binding site on a single microtubule end, each with a distinct lifetime. *In vivo* experiments, in which a lower temperature caused a reduction in microtubule growth rate, resulted in an increased decoration time but not in a different comet tail length for GFP-CLIP170 [79]. Thus, the *in vivo* and *in vitro* requirements for the turnover of microtubule-end binding sites appear to differ, perhaps because the many different +TIPs that are present in cells are able to form multiple transient connections [15], whereas single +TIPs in a purified and reconstituted *in vitro* system do not.

Growing Microtubule Ends as Platforms for Productive Protein Interactions

The search and capture model, discussed above, does not assign a function to the ends of growing microtubules until they actually contact a substrate via end-on or lateral interactions. Do cells indeed waste so much energy on random microtubule exploration, or is there more to microtubule ends? Interestingly, there are +TIPs that do not appear to directly influence microtubule dynamics (for example, see [95]), indicating that growing microtubule ends might serve more purposes than to randomly explore the cytoplasm 'in search of capture'. We have previously proposed a 'raft' model, in which protein concentration at microtubule ends allows a cascade of interactions to occur [96]. An extension of this view is not only that microtubule ends concentrate +TIPs, but that the regular pattern of the microtubule surface also orients these proteins in a specific manner when they bind the microtubule end. Binding may even induce conformational changes: for example, both EB1 and CLIP-170 have been proposed to unfold upon microtubule end binding. This combination of forced/oriented binding, increased local concentration and induced conformational changes may enhance productive protein interactions at microtubule ends. These interactions can also occur in the cytoplasm but are much less common because then proteins collide randomly. Thus, growing microtubule ends, which move through the cytoplasm of cells and are continuously remodeled themselves, can be considered to be nanoplatforms for productive protein interactions. The consequence for a cell is that the cytoplasmic concentration of certain proteins (e.g. +TIPs) can be kept low. The energy wasted by constantly assembling and disassembling microtubules is compensated by the ability to reduce the biosynthesis of several +TIPs and associated proteins. From an evolutionary standpoint, it seems simpler for proteins to acquire an SxIP motif and participate in the network of productive protein interactions at microtubule ends than to specifically increase their affinity for other proteins. The model proposed here functions optimally if the pool of productively interacting proteins 'recycles' through growing microtubule ends in a rapid manner, which, in the fast exchange model, is indeed the case.

If selected +TIPs do form productive interactions at microtubule ends, one question is whether these persist after dissociation of the interacting +TIPs from the microtubule end. If so, +TIP complexes might form at plus ends and act elsewhere in the cell. This provides a cell with a 'memory' of growing microtubules. A persistent interaction of CLIPs with other proteins, for example, could explain why CLIPs bind to the ends of growing microtubules yet function at shrinking microtubule ends. In this respect, it is interesting to note that CLIPs and CLASPs interact in different organisms [20,97,98], colocalize at microtubule ends and are both involved in local microtubule rescue [61,99]. These proteins might form a relatively stable complex at the ends of growing microtubules, dissociate, and subsequently function as a rescue complex at shrinking microtubules. Here, the CLIP–CLASP interaction functions in a positive-feedback loop, in which growing microtubules generate complexes that stimulate rescue of shrinking microtubules, leading to a local increase in the fraction of growing microtubules.

Conclusions

The *in vitro* reconstitution of the microtubule plus-end tracking phenomenon, in combination with a structural analysis of the interactions of +TIPs and improved methods to analyze +TIP interactions with microtubules, have led to a better understanding of how +TIPs accumulate at microtubule ends. Future studies should provide insights into further members of the +TIP family, the exact features of microtubule ends that are recognized by individual +TIPs, and whether plus-end tracking serves a broader purpose than to regulate microtubule dynamics and the interaction of microtubules with other structures.

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