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Title
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Permalink
https://escholarship.org/uc/item/7383504q

Journal
Trends in Cell Biology, 22(8)

ISSN
0962-8924

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Publication Date
2012-08-01

DOI
10.1016/j.tcb.2012.05.005

Peer reviewed

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Trends Cell Biol. Author manuscript; available in PMC 2013 August 01.

Published in final edited form as:
Trends Cell Biol. 2012 August ; 22(8): 418–428. doi:10.1016/j.tcb.2012.05.005.

+TIPs: SxIPping along microtubule ends

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Abstract

+TIPs are a heterogeneous class of proteins that specifically bind to growing microtubule ends. Because dynamic microtubules are essential for many intracellular processes, +TIPs likely play important roles in regulating microtubule dynamics and microtubule interactions with other intracellular structures. End-binding proteins (EBs) recognize a structural cap at growing microtubule ends, and have emerged as central adaptors that mediate microtubule plus-end-tracking of potentially all other +TIPs. The majority of these +TIPs bind EBs through a short hydrophobic SxIP sequence motif and surrounding electrostatic interactions. These recent discoveries have resulted in a rapid expansion of the number of possible +TIPs. In this review, we outline our current understanding of the molecular mechanism of plus-end-tracking and provide an overview of SxIP-recruited +TIPs.

Keywords

microtubule; microtubule dynamics; +TIPs; plus-end-tracking; EB1

+TIPs localize to growing microtubule ends

Microtubules are central to many essential processes in eukaryotic cells including accurate chromosome segregation, intracellular transport, and cell shape remodeling. Microtubules are highly dynamic filaments that stochastically switch between phases of growth and shortening (Box 1). In the late 1990s a group of proteins, now commonly called +TIPs, was discovered that associate only with growing microtubule ends in cells [1,2]. This discovery opened up previously unanticipated possibilities of microtubule regulation and function beyond microtubule-associated proteins that bind along the entire microtubule lattice, and kinesin and dynein motors that transport cargo along microtubule tracks.

In cells, +TIPs form a characteristic comet-shaped region at growing microtubule ends (Fig. 1 a), and ever since the original description of this remarkable plus-end-tracking behavior, the mechanism by which +TIPs localize to growing microtubule ends has been hotly debated. Numerous plus-end-tracking models have been proposed including co-polymerization of +TIPs with tubulin dimers and subsequent release from the microtubule lattice, motor-dependent or -independent forward translocation of +TIPs toward the growing microtubule end, as well as direct recognition of microtubule plus end-associated structures

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such as, for example, sheet-like polymerization intermediates or a cap of GTP-loaded tubulin subunits at growing ends (Fig. 1 b) [3,4].

Although some proteins such as microtubule-depolymerizing kinesins and the microtubule polymerase XMAP215 can directly associate with microtubule ends *in vitro* [5,6], EB1 family proteins have emerged as central adaptors that recruit other +TIPs to growing microtubule ends (Box 2). These end-binding proteins (EBs) are responsible for most if not all intracellular plus-end-tracking. The current review focuses on recent advances in how EBs recognize growing microtubule ends, and how EBs recruit other +TIPs to growing microtubule ends. In addition, we discuss the growing class of +TIPs that interact with EBs through recently identified SxIP sequence motifs.

EB proteins recognize a structural cap at growing MT ends

Recent data strongly suggest that the mechanism underlying EB plus-end-tracking is directly related to GTP hydrolysis-driven conformational changes at growing microtubule ends. In vitro reconstitution experiments with the fission yeast EB1 homologue Mal3 [7] first established that EB proteins and dynamic microtubules are sufficient for microtubule plus-end-tracking behavior. This was quickly followed by several reports demonstrating *in vitro* plus-end-tracking of vertebrate EBs [8-10]. Single molecule analysis further demonstrated that the dwell time of individual EB molecules on growing microtubule ends is in the order of hundreds of milliseconds, which is at least an order of magnitude shorter than the lifetime of the EB-labeled region on a growing microtubule plus end [7,8,10]. This difference immediately excludes plus-end-tracking mechanisms that depend on prolonged association of EB molecules with the growing microtubule end such as copolymerization or treadmilling. Instead, these data strongly suggest that plus-end-tracking is governed by equilibrium binding to a special structure at newly polymerized microtubule ends that is different from the remainder of the older microtubule lattice, and reflects an intrinsic structural property of growing microtubule ends. This also implies that, although time-lapse movies of plus-end-tracking show forward movement of +TIP comets, no actual transport of +TIP molecules along microtubules occurs. In addition, the characteristic fluorescence profile of EB comets at growing microtubule ends in cells and *in vitro* as well as the remarkably constant lifetime of the EB1-labeled region are consistent with a first order exponential decay of plus-end-associated high affinity EB binding sites (Fig. 1 a).

Experiments utilizing microtubules assembled in the presence of different types of GTP analogs have helped to identify these high-affinity binding sites. Although a protective layer of GTP-loaded tubulin dimers at the plus end of growing microtubules has been postulated, this GTP cap may consist of as little as one tubulin dimer per protofilament [11]. This is not consistent with the length of the EB-binding region of hundreds of binding sites. In addition, immunofluorescence with antibodies directed against the GTP-tubulin conformation does not reveal extended labeling at microtubule ends, and looks very different from the typical EB comet [12]. Finally, microtubules assembled with GMPCPP, a slowly hydrolysable GTP analogue that keeps tubulin in a conformation that closely resembles the GTP-bound state, bind EBs only with slightly increased affinity compared with GDP-microtubules [13,14]. In contrast, EB proteins bind very strongly along microtubules in the presence of BeF_3^-, which occupies the tubulin γ-phosphate binding site after GTP hydrolysis. This is generally considered to mimic the GDP/P_i transition state after GTP hydrolysis, but before dissociation of the hydrolyzed phosphate group. EBs bind with even higher affinity to GTPγS-loaded microtubules. Thus, at least in tubulin, GTPγS may also mimic the GDP/P_i conformation although this has not been directly demonstrated [14].

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Cryo-electron microscopy of the microtubule-binding domain of Mal3 bound to GTPγS-microtubules in combination with single particle reconstruction allowed the generation of a very high precision structural model of EB-binding to microtubules [15]. These exciting new data show that EBs bind microtubules in a groove between protofilaments contacting four different tubulin dimers (Fig. 1 b,c). This interface is characteristic of the B-type microtubule lattice, and does not exist along the microtubule seam (Fig. 1 b), an obligatory lattice discontinuity in microtubules assembled under physiological conditions [16,17]. Consequently, this new high resolution structure unambiguously shows that EBs do not bind along the microtubule seam. This makes sense as it is difficult to envision how seam binding could explain the exponential decay of EB binding sites at growing microtubule ends. However, EB seam binding has been observed in previous electron microscopy experiments [18], but important differences exist between these studies. The newer study uses a higher concentration of Mal3 to achieve saturated microtubule decoration, but it seems odd why this would exclude Mal3 binding from the seam. Another difference is that the newer high-resolution model was derived from microtubules decorated with the monomeric Mal3 calponin homology domain [15], whereas seam binding was observed with full-length Mal3 dimers [18]. Although EB dimerization increases the affinity for microtubules [19], which suggests simultaneous binding of both EB heads, the shape of the fluorescence profile of monomeric or dimeric EB comets on microtubule ends is indistinguishable [9]. In addition, monomeric Mal3 promotes formation of microtubules in the A-type lattice configuration that is characteristic of the microtubule seam [20]. Thus, EB dimerization cannot explain the observed discrepancy of microtubule seam binding. Ultimately, cryo-electron microscopy or other structural studies of EBs on real growing microtubule ends are needed to completely unravel the structural basis of plus-end-tracking.

Interestingly, the EB binding site appears to be perfectly located to sense GTP-hydrolysis mediated conformational changes in the β-tubulin γ-phosphate binding site. Thus, a relatively slow conformational change in the microtubule lattice that occurs after GTP hydrolysis likely forms the basis of plus-end-tracking. Intriguingly, another microtubule-associated protein, doublecortin, that does not share any obvious sequence homology with EBs binds to the same site on microtubules [21], and remarkably doublecortin can track growing microtubule ends in vitro independent of EBs [22]. Because EBs bind only weakly to the GTP-bound tubulin conformation [14], EB proteins likely do not bind to the real microtubule end, but rather to a slowly decaying region of intermediate conformation behind the GTP cap (Fig. 1 b). Whether this region corresponds to tubulin sheets that have been proposed as microtubule assembly intermediates is unclear [23]. Although sheet-like EB-bound microtubule extensions have been observed at microtubule ends at high EB1 protein concentrations [24], the high degree of variability of tubulin sheet lengths is not consistent with the highly reproducible single exponential decay of EB fluorescence observed at growing microtubule ends *in vitro* and in cells (Fig. 1 a). Although recognition of a GDP/P_i or similar GTP hydrolysis transition state provides an explanation for plus-end-tracking of EB proteins, electrostatic repulsion of the negatively charged tubulin and EB protein C-termini may also contribute to plus end specificity of full-length EBs [25].

This new structural model of GTP-hydrolysis mediated EB binding to the microtubule lattice also helps to understand EB intrinsic effects on microtubule polymerization dynamics. Although one might expect that EB-binding should stabilize microtubules, EBs appear to increase the decay of the tubulin conformation that it recognizes [14]. This suggests a faster turnover of a protective conformational cap, and could explain increased catastrophe frequencies in the presence of only EB1 *in vitro* [9,26]. At higher concentrations, EBs also increase the microtubule growth rate *in vitro* [14,26]. This could be explained if the same EB-induced conformational change increases the rate of tube closure at growing microtubule ends [26], for example by cross-linking of adjacent protofilaments,

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and as a result stimulates microtubule growth. In cells, EB effects on microtubule polymerization dynamics are more complex, and are likely mediated to a large extent by other +TIPs that are recruited to microtubule ends by EB proteins [9,24,27,28].

EB proteins recruit other +TIPs to growing microtubule ends

In vitro reconstitution experiments also demonstrated that EBs play a central role in plus-end-tracking of most, if not all, other +TIPs. All +TIPs tested so far depend on interactions with EB1 or EB3 which are necessary and sufficient to mediate plus-end-tracking of CLIP-170, MCAK, MACF, STIM1 and CLASP2 *in vitro* [8,10,29-31]. The largest group of EB-recruited +TIPs bind to EB proteins through a conserved small peptide motif, (S/T)x(I/L)P, that binds to a hydrophobic groove formed by the C-terminal helix bundle of the EB homology domain dimer (Fig. 2 b,c) [30,32,33]. The isoleucine (or leucine) and proline residues in positions 3 and 4 of this SxIP motif participate in hydrophobic interactions and are the most conserved and most important. Mutation of these residues to more polar amino acids such as asparagine eliminates EB-binding and plus-end-tracking of all +TIPs tested so far [30,31,34]. Remarkably, the SxIP consensus was initially predicted through a bioinformatics search for short sequence motifs of a relatively small set of +TIPs known at that time [35]. In the last few years the number of +TIPs containing confirmed or predicted SxIP motifs has grown significantly (Table 1). Although the serine in position 1 of the SxIP motif contributes to hydrogen bonds with conserved residues surrounding the EB hydrophobic groove [30], recent data indicate that it is not required for plus-end-tracking. Less well conserved variations of the SxIP motif can mediate plus-end-tracking, and many +TIPs contain multiple functional SxIP motifs that act in concert and increase the affinity for EB1 [31,34]. SxIP-like motifs are common suggesting many yet undiscovered proteins that may be recruited to growing microtubule ends by EBs, and functional SxIP motifs have now also been described in yeast [36]. Although in yeast microtubule motor-based transport contributes to plus end accumulation [7,37], this does not appear to be globally important for plus-end-tracking in higher eukaryotic cells. One notable exception is the transport of Adenomatous Polyposis Coli protein (APC) along microtubules by the heterotrimeric kinesin KIF17 that is required for the formation of APC clusters near a subset of microtubule ends in migrating cells [38]. The KIF17 motor domain interacts with EB1, although this interaction does not depend on a SxIP-like motif in the motor domain and it is not clear whether this interaction is direct [38].

Clearly, SxIP motifs are required for plus-end-tracking, but by themselves are not sufficient. The hydrophobic interaction surface of the SxIP motif and as a consequence most likely the binding free energy contributed by this interaction is quite small. Sequences surrounding functional SxIP motifs are interspersed with positively charged amino acids and particularly enriched in arginine residues. In contrast, the EB C-terminus is highly negatively charged, and it is reasonable to assume that strong electrostatic interactions contribute significantly to +TIP interactions with EBs (Fig. 2 b,c). However, the EB C-terminus as well as regions around SxIP motifs are predicted to be intrinsically disordered, which makes structural analysis of these interactions challenging, and these regions are not visible by X-ray crystallography [30,32]. In the case of CLASP2, molecular dynamics simulations indicate that conserved arginine residues surrounding the SxIP motifs participate in extensive salt bridge networks with negatively charged glutamates near the EB C-terminus [31]. Such coplanar arginine-glutamate salt bridges are geometrically and energetically highly favorable, and can be efficiently disrupted by phosphorylation [39]. Consequently, multisite phosphorylation by CDKs and GSK3 completely disrupt EB1-binding and CLASP2 plus-end-tracking [31,40,41]. Similar inhibition of plus-end-tracking by phosphorylation especially during mitosis has also been observed for APC, MCAK, and SLAIN2 [30,34,42], and more recently for the fission yeast Aurora kinase homologue Ipl1 [36], although Aurora

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kinase interactions with EBs appear not to be conserved in mammalian cells. Kebab, a newly described Drosophila +TIP of unknown function is absent from metaphase spindles and progressively accumulates on microtubules during anaphase [43]. This is likely due to phosphoregulation, but this has not been tested. Other +TIPs such as the spectraplakins MACF1/ACF7 are also extensively phosphorylated by GSK3 near confirmed SxIP motifs, but whether this regulates EB1-binding or plus-end-tracking has not been established [44]. Thus, EB-mediated recruitment of SxIP motif containing +TIPs depends both on weak hydrophobic interactions of the SxIP motif that determine specificity, and less specific electrostatic 'molecular Velcro' that contributes the majority of the binding free energy. These electrostatic interactions can readily be controlled and tuned by multisite phosphorylation.

A second, smaller class of EB-recruited +TIPs rely on a molecular interaction that at first glance seems entirely different. These proteins include CLIP-170, CLIP-115 and the dynein-dynactin subunit $p150_{Glued}$, and bind to EBs through a conserved CAP-Gly domain (Fig. 2 b). CAP-Gly domains are not only found in bona fide +TIPs, but occur in a number of other tubulin and microtubule-binding proteins [45]. The structure and interactions of CLIP-170 and other CAP-Gly domain containing proteins have been reviewed recently [4,45,46], and will not be covered in great detail here. Briefly, CAP-Gly domains recognize EEY/F-COO^- motifs at protein C-termini through a shallow, conserved interaction surface that consists of a basic groove and a hydrophobic patch [47,48]. CLIP-170 has two CAP-Gly domains that are not equivalent, and the EEY/F affinity of different CAP-Gly domains may depend on the number of lysine residues in the basic groove [47,49]. Most EB and α-tubulin isoforms, CLIP-170 itself and the SxIP motif containing +TIP SLAIN2 have similar C-terminal EEY/F sequence motifs [34,48,50,51]. Consequently, the C-terminal tyrosine residues of EBs and α-tubulin are required for CLIP-170 plus-end-tracking [8], and tubulin detyrosination in cells disrupts plus-end-tracking of CAP-Gly domain proteins but not of SxIP motif containing +TIPs [52]. CLIP-170 also adopts a folded, inactive conformation in which the CAP-Gly domains interact with its own C-terminus, and the CLIP-170 C-terminus can bind to the CAP-Gly domain of $p150_{Glued}$ [47,53]. Thus, CLIP-170 appears to participate in complex interaction networks with other +TIPs. Although CLIP-170 was the first protein for which plus-end-tracking was ever demonstrated [1], and CLIP-170 has since been an intense subject of study, the functional significance of these multiple potential interactions still remains mysterious. Curiously, CLIP-170 also contains a SxIP motif, but it is not known whether this contributes to plus-end-tracking. In addition to binding to the EEY/F motif, the CAP-Gly domain of $p150_{Glued}$ also forms hydrophobic contacts with the same EB hydrophobic cavity that is occupied by the SxIP motif [48,54]. This suggests an interesting parallel in the EB-mediated recruitment of CAP-Gly and SxIP motif containing +TIPs involving weak interactions with a conserved EB hydrophobic cavity and stronger electrostatic interactions with the acidic EB C-terminal tail. However, to what extent CAP-Gly and SxIP motif containing +TIPs cooperate or compete for EB binding has not been tested.

SxIP motif containing +TIPs are functionally diverse

As the number of EB-recruited, SxIP motif containing +TIPs continues to grow (Table 1), it is becoming evident that these proteins are structurally highly heterogeneous. Other than one or multiple SxIP motifs in positively charged, intrinsically disordered regions, there is very little structural similarity between these proteins. This structural diversity strongly suggests that +TIP functions are also highly diverse. Although certain functional similarities between different +TIPs are beginning to emerge, many +TIPs will have overlapping functions or not fit into a clearly defined category.

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Microtubule plus end adaptors

The idea of search-and-capture of intracellular structures by growing microtubules was initially proposed as a mechanism by which dynamic microtubules find kinetochores during mitosis. Search-and-capture in its simplest form in which growing microtubules randomly hit intracellular targets is too inefficient on a cell biological timescale [55]. However, certain +TIPs may function as facilitators of microtubule interactions with other intracellular structures or organelles. Such +TIPs are targeted through binding domains independent of microtubules, and provide a receptor with which EB-covered growing microtubule ends can readily interact. Although all +TIPs track growing microtubule ends in cells when overexpressed as GFP fusions proteins, some +TIPs are much more abundant on other intracellular structures. It is often difficult to detect the endogenous protein on microtubule ends by immunofluorescence suggesting that the real function of these proteins is not primarily to track growing microtubule ends, but indeed to mark certain intracellular structures as microtubule plus end interaction sites. We propose that EB1 generates a sticky surface near growing microtubule ends that facilitates binding to such microtubule capture sites.

Three different types of +TIPs, CLASPs, the spectraplakin proteins MACF1/ACF7, and APC participate in microtubule capture and stabilization near the leading edge of migrating cells. Both CLASPs and spectraplakins localize to membrane-associated structures that may represent microtubule capture sites [40,56,57]. Genetic knock-out or RNAi-mediated depletion of CLASPs or ACF7 result in remarkably similar microtubule phenotypes [40,58-60]. In both cases, the density of microtubules oriented toward the front of migrating cells is decreased, and microtubules appear curly and less organized. CLASPs and MACF1/ACF7 also interact in biochemical assays [56,61], and have direct, EB-independent microtubule-binding activity [44,58,62]. In addition, EB1-binding as well as microtubule-binding of both proteins is inhibited by multisite phosphorylation by GSK3 [31,40,44]. Because GSK3 is thought to be inactivated in the front of migrating cells [63], a gradient of CLASP and MACF1/ACF7 phosphorylation could increase the microtubule capture activity of these proteins toward the leading edge of migrating cells. This is consistent with a GSK3 phosphorylation-mediated gradient of CLASP microtubule-binding activity in migrating epithelial cells in which CLASPs track microtubule plus ends in the cell interior but bind along microtubules in the cell periphery [40,62]. A similar gradient of microtubule binding activity between cell interior and periphery has recently been demonstrated for the Drosophila spectraplakin, Short Stop, but it is not known whether this is regulated by phosphorylation [60]. Together these similarities suggest that CLASPs and MACF1/ACF7 function in the same microtubule capture pathway, but the functional relationship of these two +TIPs during cell migration is not understood. APC also localizes to clusters in the front of migrating cells that appear distinct from CLASPs and MACF1/ACF7 [38,61-64], and APC has been proposed to participate in microtubule capture pathways [63,65]. Although APC phosphorylation of predicted GSK3 sites near the SxIP motif inhibits binding to EB1 [30], recent data suggest that phosphoregulation of APC cluster formation and microtubule binding is complex and integrates multiple signaling pathways [66].

Several +TIPs including CLASPs and APC also localize to the kinetochore [67,68], indicating that the same +TIPs can function as microtubule capture sites in different cellular contexts. CLASPs may be especially multi-functional and also associate with the Golgi apparatus through the trans-Golgi network structural golgin GCC185 [69]. RNAi-mediated CLASP depletion results in fragmentation of the Golgi apparatus and CLASP-mediated microtubule interactions with Golgi membranes are required for reassembly of the Golgi apparatus after mitosis [70]. However, it remains to be seen whether this is directly related to CLASP-mediated microtubule interactions with Golgi membranes or whether this is a more indirect effect of a disrupted microtubule cytoskeleton in CLASP-depleted cells.

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Another example of an organelle-associated +TIP is STIM1. STIM1 is an integral endoplasmic reticulum (ER) membrane protein involved in ER calcium homeostasis. However, STIM1 also binds to EB1 and is required for ER tubule extension by attaching the ER membrane to growing microtubules [71]. Because the ER surface area is very large, stochastic interactions with growing microtubule ends may be sufficient for a simple search-and-capture mechanism in this case. Whether STIM1-mediated ER-microtubule association is sufficient for ER tubule extension or whether this requires cooperation of a motor protein remains to be determined. Proteins such as melanophilin, that mediates interactions of growing microtubules with melanosomes [72], or p140CAP that may capture microtubules in dendritic spines [73], could also be considered in this category.

Microtubule dynamics regulators

Because of their localization to growing microtubule ends, +TIPs are in a prime location to regulate microtubule polymerization dynamics. However, it is often unclear whether +TIP effects on microtubule dynamics are direct or indirect. For example, one would expect that microtubule capture by CLASPs, MACF1/ACF7, or APC may indirectly protect microtubules from depolymerization. Consequently, effects on microtubule polymerization have been described for many +TIPs. However, the primary function of a subset of +TIPs may be the regulation of microtubule dynamics.

MCAK belongs to the kinesin-13 family of microtubule motors that do not move along microtubules but instead enzymatically promote microtubule depolymerization [6]. Although MCAK has an intrinsically high affinity for microtubule ends [6], MCAK also plus-end tracks through SxIP-mediated interaction with EBs [30,42,74]. In addition, TIP150, another SxIP motif containing +TIP further promotes the recruitment of MCAK to growing microtubule ends [75], but it remains unclear to what extent direct or indirect EB interactions control MCAK depolymerase activity. Interestingly, KIF18B, a microtubule depolymerizing kinesin from a different branch of the kinesin family tree, also tracks microtubule plus ends in an EB1-dependent manner [76,77], and KIF18B is required to limit the length of astral microtubules during mitosis [76]. In addition, MCAK and KIF18B appear to be physically and functionally connected, and cooperate in the regulation of mitotic spindle microtubule stability [77].

The microtubule depolymerizing activity of MCAK is counterbalanced by microtubule polymerases of the XMAP215/ch-TOG family [6]. The TOG domains in XMAP215 are thought to catalytically deliver tubulin subunits to the growing microtubule end, and at least *in vitro* XMAP215 tracks growing microtubule ends independent of EB proteins [5]. In interphase, the SxIP motif containing +TIP SLAIN2, contributes to XMAP215 localization to growing microtubule ends, and this is required for processive microtubule growth in the cell interior [34]. Because EB proteins are required for SLAIN2 plus-end-tracking, this is consistent with earlier findings that simultaneous depletion of all mammalian EB family proteins results in a similar microtubule growth defect [9]. However, it is unclear why SLAIN2 plus end localization and thus presumably XMAP215-mediated stimulation of microtubule polymerization is inhibited during mitosis [34] when microtubule growth rates are increased although this may indicate differences in microtubule regulation between interphase and mitotic cells. Similarly, the Drosophila XMAP215 homologue Mini spindles tracks microtubule plus ends in an EB-dependent manner [78], which requires interaction with the SxIP motif containing +TIP Sentin that tracks microtubule ends in both interphase and mitosis [79]. No clear sequence homology exists between SLAIN2 and Sentin, and it remains to be tested to which extent these proteins are functional homologues although this does indicate strong evolutionary conservation of EB1-mediated XMAP215/ch-TOG plus-end-tracking. CLASPs also contain several TOG-like domains [19], and the yeast CLASP homologue has a microtubule rescue promoting activity [80]. However, the domain structure

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of mammalian and yeast CLASP is quite dissimilar, and it remains to be determined whether mammalian CLASPs can directly act as rescue factors and to which extent proposed mechanistic similarities exist between XMAP215 and CLASPs [81].

Concluding remarks

The discovery of the microtubule plus-end-tracking phenomenon makes a strong case for the vital importance of curiosity-driven, basic cell biology research. Over the last decade, +TIPs have evolved from a cell biological oddity into a large and diverse group of proteins with many potential and largely unexplored connections to human disease (Table 1). EB-recruited +TIPs are required for epithelial remodeling in a 3D tissue culture system [28], but future challenges will be to understand to what extent plus-end-tracking contributes to the proposed core functions of specific proteins and how this impacts normal development and pathological processes. For example, how microtubule plus-end-tracking impacts APC's well-established role in the Wnt signaling pathway is not understood, and it has been proposed that the APC C-terminus that binds EB1 and is required for plus-end-tracking is not relevant for the progression of colon cancer [82]. Similarly, the interaction of melanophilin with EB1 has been postulated to participate in the off-loading of melanosomes from microtubule ends [72], but plus-end-tracking appears to be dispensable for intracellular melanosome dynamics in tissue culture cells [83].

Nevertheless, EBs have emerged as central adaptors that mediate binding of possibly all other +TIPs to growing microtubule ends. A large amount of biochemical data suggests additional interactions between many EB-recruited +TIPs [4]. The functional relevance as well as spatial and temporal regulation of such proposed +TIP interaction networks remain important questions for future research, and we are only beginning to understand the highly complex phosphoregulation of +TIP interactions. With the ongoing discovery of new EB-recruited +TIP proteins, growing microtubule plus ends continue to be an exciting place. Elucidating the molecular function of these novel +TIPs and why they need to be at microtubule ends will certainly keep cell biologists busy for many more years.

Acknowledgments

We thank Sarah Gierke and the reviewers for insightful comments on the manuscript. This work was supported by National Institutes of Health grant R01 GM079139 to T.W., and was conducted in a facility constructed with support from the Research Facilities Improvement Program grant C06 RR16490 from the National Center for Research Resources of the National Institutes of Health.

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Box 1: Microtubule structure and dynamics

Microtubules are highly dynamic, cylindrical polymers of α/β-tubulin dimers [16]. In cells, each microtubule typically consists of thirteen parallel protofilaments [17], although microtubules with more or less protofilaments can be polymerized *in vitro* and exist in specialized microtubule structures. Lateral interactions between protofilaments are largely homotypic, i.e. an α-tubulin subunit interacts laterally with neighboring α-tubulin subunits. However, a slight helical pitch between adjacent protofilaments results in a longitudinal shift of three tubulin monomers between the first and the thirteenth protofilament. Because of this arrangement, which is often referred to as B-type microtubule lattice or 3-start helix, microtubules assembled under physiological conditions have at least one lattice discontinuity in which lateral interactions occur between α and β-tubulin subunits. This discontinuity is referred to as the microtubule seam (Fig. 1 b).

In each protofilament, α/β-tubulin dimers are arranged in a head-to-tail fashion, which results in an intrinsic polarity of the microtubule filament. In cells, only the plus end, which is terminated by β-tubulin subunits, displays a non-equilibrium behavior referred to as dynamic instability in which individual microtubules stochastically switch between states of polymerization (growth) and depolymerization (shortening). The transitions between these states are commonly called catastrophe and rescue. It is important to remember that because of the refraction limit of the light microscope, the smallest observable change in microtubule length corresponds to association or dissociation of many hundred α/β-tubulin dimers. Thus, while this definition of microtubule dynamic instability is useful as an analytic tool, microtubule polymerization dynamics at the molecular scale are more complex [11].

Microtubule dynamic instability is driven by a conformational change of the α/β-tubulin dimer as a consequence of β-tubulin-associated GTP hydrolysis. Only GTP-loaded tubulin dimers can add onto a growing microtubule end. Incorporation into the microtubule lattice triggers GTP hydrolysis in the adjacent β-tubulin, and most β-tubulin in the microtubule lattice is in the GDP-bound form. However, growing microtubule ends are thought to be capped by a short layer of GTP-bound β-tubulin. Because GTP-tubulin dimers are straighter than GDP-tubulin, loss of a protective cap at the microtubule plus end allows strain in the lattice to trigger catastrophic depolymerization. This model is consistent with structural differences in polymerizing and depolymerizing microtubule ends that have been observed by cryo-electron microscopy. Growing ends terminate in a relatively straight sheet, while depolymerizing ends display highly curled and peeling protofilaments.

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Box 2: End binding proteins

EB1 was initially identified as a binding partner of the C-terminus of the adenomatous polyposis coli (APC) tumor suppressor protein. Subsequently, EB1 homologues were identified in all other eukaryotic cells including yeast and plants. Several years later EB1 was found to associate specifically with growing microtubule plus ends by live cell microscopy [2], and EB became synonymous with microtubule end-binding. Most mammalian cells express at least three EB1 related proteins. While EB1 and EB3 are considered functionally similar, the role of EB2 is less understood and independent plus-end-tracking has not been demonstrated [50]. There is also a high variability in the number of EB protein encoding genes in different species. For example, dogs have more than ten potential EB1 genes [28], although the significance of this is not understood. In this review, we generically refer to EB1 family proteins as EBs.

EBs form homo- or heterodimers [103]. The globular, N-terminal calponin homology (CH) domain is required for microtubule binding. In contrast, the C-terminal EB homology (EBH) domain mediates dimerization, and forms an elongated coiled coil that folds back on itself and terminates in a short 4-helix bundle. The base of this bundle constitutes the two hydrophobic grooves that bind the SxIP sequence motif (Fig. 2 c) [30]. The acidic, unstructured C-terminal tail is not resolved in crystal structures and terminates in a conserved EEY/F-COO^- motif that is very similar to the tubulin C-terminus. The CH and EBH domains are connected through a flexible linker, and although the structure of both domains has been independently solved by X-ray crystallography [32,104], only recently small angle X-ray scattering (SAXS) combined with single particle electron microscopy has yielded a low resolution model of the entire EB1 molecule [25]. Surprisingly, although EB1 is a homodimer, the molecule is highly asymmetric. The C-terminal rod-like coiled coil is more closely associated with one of the CH domains and protrudes from the microtubule-binding domains at an angle of almost 45° (Fig. 2 a). In addition, the spacing between the two CH domains is significantly less than the 8 nm distance between potential EB binding sites along a microtubule protofilament. Whether the EB dimer could instead bridge the shorter distance between binding sites on adjacent protofilaments is a currently unresolved question.

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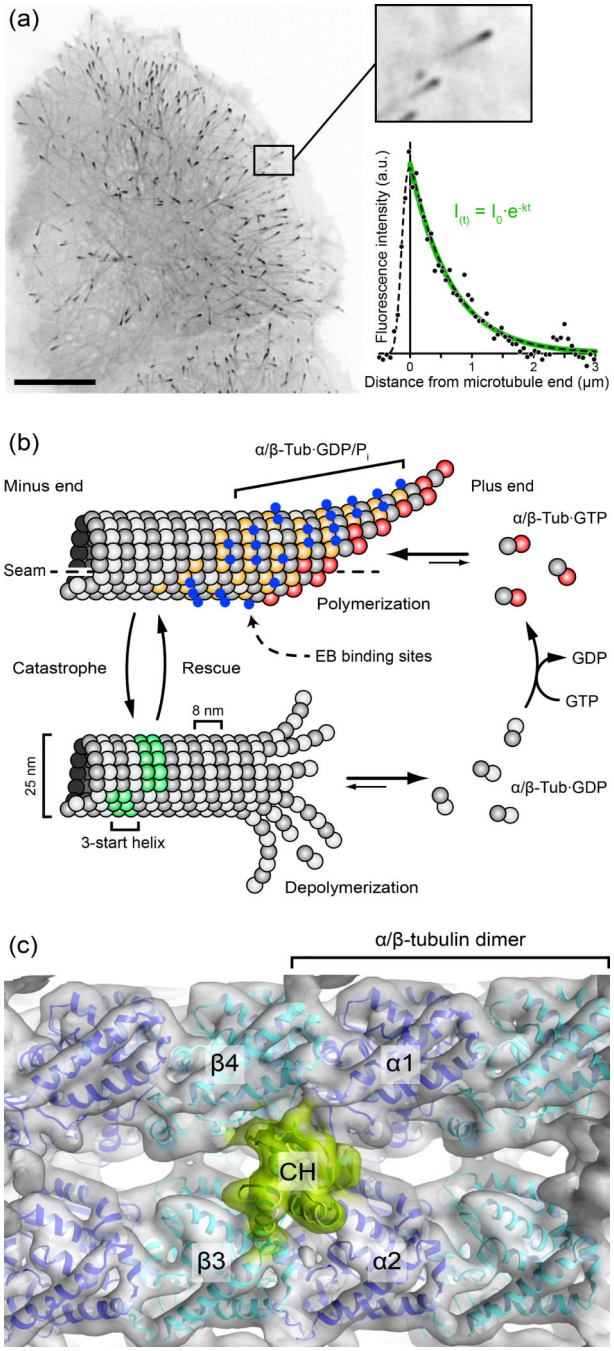

Figure 1.

EB proteins recognize an intermediate GTP hydrolysis state at growing microtubule ends. (a) Contrast-inverted image of a tissue culture cell expressing EB1-EGFP illustrating the characteristic +TIP comet shape at growing microtubule ends. Scale bar is 10 μm. The inset shows the indicated region at the cell edge at higher magnification, and the graph below is the fluorescence intensity profile of the topmost EB1 comet in the inset. The dashed line is an exponentially modified Gaussian fit that takes into account the blur introduced by the microscope point spread function [31]. The green line is a first order exponential fit of only the decay phase. (b) Diagram highlighting central features of microtubule structure and dynamic instability. The structural cap at growing microtubule ends likely consists of a layer

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of GTP-loaded tubulin dimers at the very end that is followed by a longer section of tubulin dimers in an intermediate GTP hydrolysis state, possibly GDP/P_i. High affinity EB binding sites at the interface of four tubulin dimers in a GDP/P_i state near the growing microtubule end are indicated in blue. These sites do not exist along the microtubule seam. The first order decay of these binding sites determines the exponential fluorescence profile of +TIPs at growing microtubule ends. The remainder of the lattice is mostly GDP-tubulin. (c) High resolution model of the microtubule-bound Mal3 calponin homology (CH) domain reconstructed from cryo-electron microscopy data. The CH domain binds at the interface of four tubulin dimers in the B-type microtubule lattice. Image reproduced with permission from [15].

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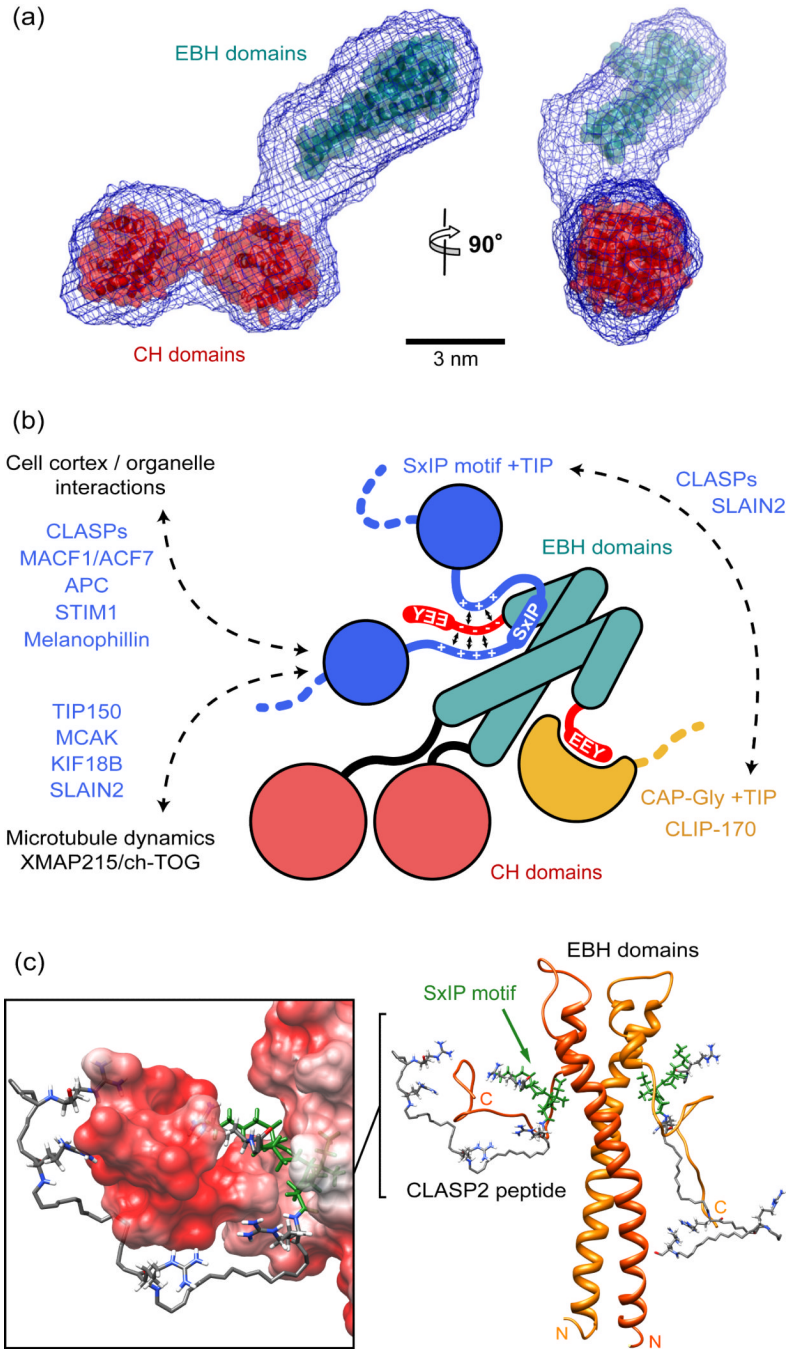

Figure 2.

EB protein structure and interactions. (a) Model of the EB1 homodimer, reproduced with permission from [25]. Crystal structures of the calponin homology (CH) and EB homology (EBH) domains were manually fitted into the envelope shape calculated from SAXS data (blue mesh). (b) Diagram of interactions between EB1 and SxIP- or CAP-Gly-containing +TIPs. Dashed arrows indicate interactions of SxIP motif containing +TIPs with other proteins. Some SxIP motif +TIPs such as CLASPs and SLAINs interact with CLIP-170, but the functional significance of these interaction networks is incompletely understood. (c) Interaction of the EBH domains with SxIP motif containing CLASP2 peptides. The structure was derived from homology modeling and molecular dynamics simulations [31]. Only the

backbone is shown for CLASP2 peptides except for the SxIP motif (green) and nearby arginine residues. The inset shows electrostatic surface coloring of the intrinsically disordered C-terminal part of the EBH domain highlighting the interaction of positively charged arginine residues with the negatively charged EBH C-terminus.

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Table 1

EB1-recruited +TIPs that contain confirmed or potential SxIP motifs.

Protein	SxIP motifs	Phosphorylation near SxIP motifs	Uniprot Identifier	Proposed functions
+TIPs with confirmed SxIP motifs				
CLASP1, CLASP2	SKIP SRIP [30,31]	CDKs, GSK3 [31,40,41]	Q7Z460 O75122	Microtubule capture [40,57,58], dynamics [80], and nucleation at the Golgi apparatus [69]
MACF1/ACF7	SKIP [30]	GSK3 [44]	Q9UPN3	Microtubule capture [56,59,84]
APC	SQIP [30]	CDKs, GSK3 (phosphorylation not confirmed *in vivo*) [30,32]	P25054	Microtubule capture [63,64,85,86]; mutated in colon adenocarcinoma [87]
STIM1	TRIP [30]	Mitotic phosphorylation [88], mass spectrometry	Q13586	ER-microtubule interactions [71]; activator of store-operated Ca^{2+} entry; mutated in certain immunodeficiencies [89]
MCAK	SKIP [30]	Aurora B [42,90]	Q99661	Microtubule Depolymerase [6]
Kif18B	SFLP SSLP [76,77]	likely, mass spectrometry	Q86Y91	Microtubule Depolymerase [6]
SLAIN2	GGIP SAIP SGLP GGIP RSLP [34]	Mitotic phosphorylation [34]	Q9P270	Microtubule dynamics; recruits XMAP215/ch-TOG to microtubule plus ends [34]
Sentin/SSP2 (*Drosophila*)	TGIP [91]	Not determined	Q9VUA5	Microtubule dynamics; recruits XMAP215/ch-TOG homologue to microtubule plus ends [91]
CDK5RAP2	SRLP [92]	Not determined	Q96SN8	Centrosome maturation; mutated in autosomal recessive primary microcephaly [93]
Kebab (*Drosophila*)	TKIP TCIP [43]	Not determined	Q9VQ69	Kinetochore component; unknown function [43]
Ipl1 (*S. cerevisiae*)	SKIP SKIP [36]	CDK1 [36]	P38991	Yeast Aurora kinase homologue
+TIPs with probable SxIP motif				
Melanophilin [72]	SNLP	Not determined	Q9BV36	Melanosome transport; mutated in Griscelli syndrome [94]
p140CAP [73]	TSIP	Not determined	Q9C0H9	Microtubule interactions in dendritic spines [73]; Src kinase regulator
MTUS2/TIP150 [75]	SRLP SNLP SRLP SLLP	likely, mass spectrometry	Q5JR59	Microtubule dynamics; recruits MCAK to microtubule plus ends[75]
FOP [95]	SKIP	likely, mass spectrometry	Q95684	Centrosome microtubule anchoring [95]; mutated in certain myeloid cancers [96]
NAVIGATOR1 (2, 3) [97,98]	SRIP SGIP SLIP	Not determined	Q8NEY1 (Q8IVL1, Q8IVL0)	Neurite outgrowth
DRhoGEF2 [99]	SKIP	Not determined	Q44381	Rho GTPase signaling [99]
Potential SxIP motif-containing EB-binding proteins; unconfirmed plus-end-tracking activity				
PSRC1/DDA3 [100]	SAIP	Not determined	Q6PGN9	Mitotic regulator of MCAK [101]

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Protein	SxIP motifs	Phosphorylation near SxIP motifs	Uniprot Identifier	Proposed functions
Plexin-B3 [102]	SGIP	Not determined	Q9ULL4	Axon guidance