Mammalian end binding proteins control persistent microtubule growth

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Introduction

Microtubules (MTs) are intrinsically polar filaments with two structurally and functionally distinct ends, the plus- and the minus-end (Desai and Mitchison, 1997; Howard and Hyman, 2003). In cells, MT minus-ends are predominantly stable and often associated with the MT organizing center, whereas MT plus-ends spontaneously switch between phases of growth and shrinkage (Desai and Mitchison, 1997; Howard and Hyman, 2003). Growing MTs accumulate at their plus-ends multiple structurally unrelated factors collectively termed MT plus-end tracking proteins, or +TIPs (Schuyler and Pellman, 2001; Akhmanova and Steinmetz, 2008).

The most conserved and ubiquitous +TIPs are end binding proteins (EBs) (Tirnauer and Bierer, 2000). These are relatively small dimeric proteins which contain an N-terminal calponin homology (CH) domain, responsible for the interaction with MTs, a linker region of unknown function, and a C-terminal coiled coil domain that extends into a four-helix bundle, required for dimer formation (for review see Akhmanova and Steinmetz, 2008). It has been proposed that dimerization is an essential feature required for the plus-end tracking behavior of the EBs and other +TIPs (Slep and Vale, 2007). EBs terminate with a flexible acidic tail containing the C-terminal EEY/F sequence, which is important for self-inhibition and binding to known MT regulators. This probably occurs through their action on microtubule ends, because catastrophe suppression does not require the EB domains needed for binding to known EB partners.

End binding proteins (EBs) are highly conserved core components of microtubule plus-end tracking protein networks. Here we investigated the roles of the three mammalian EBs in controlling microtubule dynamics and analyzed the domains involved. Protein depletion and rescue experiments showed that EB1 and EB3, but not EB2, promote persistent microtubule growth by suppressing catastrophes. Furthermore, we demonstrated in vitro and in cells that the EB plus-end tracking behavior depends on the calponin homology domain but does not require dimer formation. In contrast, dimerization is necessary for the EB anti-catastrophe activity in cells; this explains why the EB1 dimerization domain, which disrupts native EB dimers, exhibits a dominant-negative effect. When microtubule dynamics is reconstituted with purified tubulin, EBs promote rather than inhibit catastrophes, suggesting that in cells EBs prevent catastrophes by counteracting other microtubule regulators. This probably occurs through their action on microtubule ends, because catastrophe suppression does not require the EB domains needed for binding to known EB partners.

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The most conserved and ubiquitous +TIPs are end binding proteins (EBs) (Tirnauer and Bierer, 2000). These are relatively small dimeric proteins which contain an N-terminal calponin homology (CH) domain, responsible for the interaction with MTs, a linker region of unknown function, and a C-terminal coiled coil domain that extends into a four-helix bundle, required for dimer formation (for review see Akhmanova and Steinmetz, 2008). It has been proposed that dimerization is an essential feature required for the plus-end tracking behavior of the EBs and other +TIPs (Slep and Vale, 2007). EBs terminate with a flexible acidic tail containing the C-terminal EEY/F sequence, which is important for self-inhibition and binding to various partners (Hayashi et al., 2005; Akhmanova and Steinmetz, 2008). Through their C-terminal sequences, EBs interact with most other known

Abbreviations used in this paper: CH, calponin homology; DIC, differential interference contrast; EB, end binding protein; HA, hemagglutinin; IP, immunoprecipitation; MT, microtubule; +TIPs, microtubule plus-end tracking proteins; TIRFM, total internal reflection fluorescence microscopy.
+TIPs and recruit many of them to the growing MT ends (Akhananova and Steinmetz, 2008).

Recently, the plus-end tracking phenomenon has been reconstituted in vitro using purified +TIPs from fission yeast (Bieling et al., 2007) and vertebrates (Bieling et al., 2008; Dixit et al., 2009). EB1 and its yeast homologue, Mal3, were able to accumulate at the growing MT ends on their own, in the absence of other factors. Moreover, EB1 and Mal3 were required for the plus-end tracking behavior of other +TIPs, confirming the idea that EBs form the core of plus-end tracking complexes. Measurements of EB protein dynamics showed that they exchange very rapidly at the growing MT ends (Busch and Brunner, 2004; Bieling et al., 2007, 2008; Dragestein et al., 2008; Dixit et al., 2009), suggesting that they recognize some specific structural feature associated with MT polymerization.

Inactivation of EBs has profound effects on MT organization and dynamics. EBs are involved in MT anchoring at the centrosome (Rehberg and Graf, 2002; Louie et al., 2004; Yan et al., 2006) and cilium formation (Schröder et al., 2007). The effects of the EBs on MT plus-end dynamics vary between different experimental systems. In budding yeast and in cultured *Drosophila* cells EB1 homologues make MTs more dynamic and decrease the time MTs spend pausing (Tirnauer et al., 1999; Rogers et al., 2002; Wolyniak et al., 2006). In *Xenopus* extracts EB1 stimulates MT polymerization, promotes MT rescues, and inhibits catastrophes (Tirnauer et al., 2002). Also, the fission yeast homologue of EB1 inhibits catastrophes and stimulates the initiation of MT growth (Busch and Brunner, 2004). However, when reconstituted with purified tubulin, both EB1 and Mal3 appeared to stimulate not only rescues but also catastrophes, suggesting that they alter MT end structure, possibly by increasing the size of tubulin sheets (Bieling et al., 2007; Vitre et al., 2008). It should be noted that another study on in vitro reconstitution of MT dynamics with purified tubulin did detect catastrophe suppression by EB1 (Manna et al., 2007), while yet another study observed no effect of EB1 (Dixit et al., 2009). Structural studies suggest that the EBs probably act by enhancing lateral interactions between individual protofilaments and may affect MT lattice structure (Sandblad et al., 2006; des Georges et al., 2008; Vitre et al., 2008).

Despite these significant advances, important questions remain unanswered. Does the in vivo modulation of MT dynamics by EBs depend on their interactions with their numerous partners? Is MT tip association of the EBs sufficient to affect MT dynamics? Is dimerization important for different aspects of EB function? Do all EBs act in the same way? The latter question is particularly important for mammalian cells, which express three members of the EB family—EB1, EB2 (RP1), and EB3 (EBF3) (Juwana et al., 1999; Su and Qi, 2001; Komarova et al., 2005). So far, functional analyses have been mostly focused on EB1: depletion of EB1 in mouse fibroblasts promoted MT pausing and decreased the time MT spent in growth (Kita et al., 2006). Another study demonstrated the involvement of EB1 in formation of stable MTs (Wen et al., 2004). The other two EBs, EB2 and EB3, remained largely ignored, although analysis in differentiating muscle cells did point to a specific function of EB3 (Straube and Merdes, 2007).

Here we show that MT catastrophe suppression is the major activity of mammalian EBs in cultured cells; EB2 is much less potent compared with EB1 and EB3. This functional difference is, at least in part, due to substitutions in the CH domain. Furthermore, we show that the EBs can heterodimerize through their C-terminal part; we use this property to generate a dominant-negative mutant to confirm the results of protein depletion experiments. Finally, we demonstrate that MT plus-end tracking, dimerization, and partner binding activities of the EBs can be separated; we make use of this finding to dissect the EB domains required for catastrophe inhibition.

**Results**

**Mammalian EBs compete with each other for MT tip accumulation**

To address the common and distinct functions of mammalian EBs we determined how they affect the distribution of each other. We used our previously developed short hairpin (sh) RNA-expressing vectors to deplete EB1, EB2, and EB3 in CHO-K1 cells (Komarova et al., 2005). In control CHO-K1 cells EB1 and EB3 antibodies stained ∼2 μm-long comet-like structures with a peak of intensity near the MT ends (Fig. 1 A, a). In contrast, EB2 was distributed more evenly over the MT lattice and showed only a very slight accumulation at the tip (Fig. 1 A, a). This was not due to competition between EB antibodies because the same result was observed when EB2 alone was stained (Fig. S1 B, available at http://www.jcb.org/cgi/content/full/jcb.200807179/DC1). Depletion of EB1 had no effect on EB3 localization; however, it led to redistribution of EB2 to the outmost MT ends (Fig. 1 A, a'). Depletion of either EB2 or EB3 had no effect on the distribution of the remaining EBs (Fig. 1 B and C, b, c). Simultaneous depletion of EB1 and EB3 caused a redistribution of EB2 similar to that induced by the depletion of EB1 alone (Fig. 1 D, d). These observations demonstrate that the EBs accumulate at MT tips independently of each other and suggest that EB2 might be competed by EB1 and EB3 from the outmost MT tips. EB1 appears to be expressed in CHO-K1 cells at higher levels than EB3 (unpublished data), which probably explains why the single depletion of EB1, but not EB3, had an effect on EB2 localization.

**Simultaneous depletion of EB1 and EB3 increases MT catastrophe frequency in the internal cytoplasm**

We examined the roles of EBs in controlling MT dynamics by expressing YFP-tagged +TIP CLIP-170 to visualize MT growth or by microinjecting cells with Cy3-labeled tubulin. To achieve highly efficient and uniform protein depletion, shRNA vectors were introduced into cells by nuclear microinjection. In control cells, MTs grew persistently from the centrosome to the cell margin where they underwent frequent transitions between growth and shortening, and the distribution of the growing plus-ends along the cell radius was steeply ascending, in agreement with previously published data (Fig. 2 , A and C–E, Table I) (Komarova et al., 2002b). Depletion of single EB species had no effect on persistent MT growth (Table I; unpublished data for
EB1 and EB3 were not required for MT rescue in CHO-K1 cells. The acidic tail of EB1 required for partner binding is not needed for catastrophe suppression. To support the specificity of our shRNA-based assays we performed rescue experiments with EB1 constructs insensitive to shRNA. We co-microinjected the cells with a plasmid encoding YFP-CLIP-170 together with a vector co-expressing shRNAs against EB1 and EB3 and a rescue construct encoding either the full-length EB1 or EB1\(\text{N}H9004\)Ac (an EB1 deletion mutant lacking the last 18 amino acids). For the full-length EB1 it was impossible to assess the efficiency of substitution of the endogenous proteins with the rescue construct; this was, however, possible for EB1\(\text{N}H9004\)Ac by using the tail-specific EB1 antibody KT51, which does not react with EB1\(\text{N}H9004\)Ac but does detect the endogenous EB1. By combining it with the antibodies that detect total EB1 and EB3 pools, we could show that in cells co-microinjected with the EB1/EB3 shRNA vector and the EB1\(\Delta\)Ac mutant, the levels of

EB2 and EB3 depletion. Next, we attempted to deplete all EBs but found that complete knockdown of all three EBs was incompatible with cell viability (unpublished data). However, EB1 and EB3 could be efficiently depleted simultaneously: 3 d after shRNA microinjection the accumulation of EB1 and EB3 at the MT tips was uniformly diminished by \(\sim 90\%\) (Fig. S2 A). In such cells, YFP-CLIP-170 signal at MT tips was diminished as described previously (Komarova et al., 2005; Fig. 2 B), but it could still be used as a reliable marker of growing MT ends, as the parameters of MT growth obtained with YFP-CLIP-170 were very similar to those obtained using subtracted images of Cy3-tubulin (Table I). MT growth rate remained unchanged in these conditions; however, the length of microtubule growth episodes was decreased by a factor of 2 and only 30\% of MT ends were concentrated near the cell margin (Fig. 2, C–E and Videos 1 and 2; available at http://www.jcb.org/cgi/content/full/jcb.200807179/DC1; and Table I). Furthermore, MTs underwent catastrophe events in internal cell regions and not just at the cell margin; the catastrophe frequency increased 10-fold (Table I). MT growth phases were often interrupted by pauses followed by shortening (Fig. S2, C and D; Videos 1 and 2). By contrast, MTs in control cells showed hardly any pauses in internal cytoplasm (Fig. S2, B and D; Videos 1 and 2; Table II). Other parameters of MT plus-end dynamics were not significantly affected (Table II); for instance, MT rescue frequency remained unchanged. These data indicate that EB1 and EB3 are not required for MT rescue in CHO-K1 cells.

**The acidic tail of EB1 required for partner binding is not needed for catastrophe suppression.**

To support the specificity of our shRNA-based assays we performed rescue experiments with EB1 constructs insensitive to shRNA (Komarova et al., 2005). We co-microinjected the cells with a plasmid encoding YFP-CLIP-170 together with a vector co-expressing shRNAs against EB1 and EB3 and a rescue construct encoding either the full-length EB1 or EB1\(\Delta\)Ac (an EB1 deletion mutant lacking the last 18 amino acids). For the full-length EB1 it was impossible to assess the efficiency of substitution of the endogenous proteins with the rescue construct; this was, however, possible for EB1\(\Delta\)Ac by using the tail-specific EB1 antibody KT51, which does not react with EB1\(\Delta\)Ac but does detect the endogenous EB1 (Komarova et al., 2005). By combining it with the antibodies that detect total EB1 and EB3 pools, we could show that in cells co-microinjected with the EB1/EB3 shRNA vector and the EB1\(\Delta\)Ac mutant, the levels of
Figure 2. Simultaneous depletion of EB1 and EB3 disrupts persistent MT growth. (A and B) Time-lapse sequence of YFP-CLIP-170 in cells expressing shRNAs either to luciferase (control) (A) or to EB1 and EB3 (B). Images were acquired every 2 s. Three tips of CLIP-170 “comets” are indicated by an arrow, an arrowhead, and a black-and-white arrowhead. Projection analysis (20 successive frames) and diagrams of trajectories of individual CLIP-170 comets illustrate episodes of uninterrupted MT growth. The centrosome region is indicated by a dashed circle; the cell border is shown by a dotted line. Time in seconds is in the top right corner. Bar, 10 μm. (C and D) Histograms of MT growth rates and the lengths of YFP-CLIP-170 tracks in control (luciferase) and EB1/EB3 shRNA-expressing cells. (E) Distribution of the growing MT plus-ends along the cell radius in control and EB1/EB3-depleted cells; error bars indicate SD. The cell radius was divided into five zones (each zone was a 0.2 fraction of the cell radius) and the number of YFP-CLIP-170-positive tips were scored for each zone (~600 MT tips in 8–12 cells for each condition). The results are represented as percentage of the MTs within each zone where 100% is a total number of the scored growing plus-ends in the cell. (F and G) Rescue of EB1/EB3 depletion by EB1ΔAc. (F) EB1/EB3-depleted cells expressing EB1ΔAc are indicated by dotted lines. EB3 was detected with rabbit antibodies (green in overlay); EB1 was stained with the mouse antibody from BD Biosciences, which detects both the endogenous EB1 and EB1ΔAc (left panel; red in overlay); and the rat antibody KT51 (Absea), specific for the acidic tail of EB1, was used to stain the endogenous EB1 but not EB1ΔAc (right panel; blue in overlay). (G) Intensity distribution shows that the levels of endogenous EB1 and EB3 in EB1ΔAc-rescued cells were negligible (green and blue lines in panel g’); level of EB1ΔAc (red line in panel g’”) was similar to the level of endogenous EB1 (red line in panel g”). (H) Time-lapse sequence of YFP-CLIP-170 in the cells coexpressing shRNAs to EB1 and EB3 and EB1ΔAc rescue construct; images were acquired every 2.5 s. Tips of CLIP-170 comets are indicated by white and black-and-white arrowheads. Projection analysis and trajectories of individual YFP-CLIP-170 comets are generated in the same way as in panel A. Time in seconds is in the top right corner. Bar, 10 μm.
Because we could not achieve a complete knockdown of all EB species, we sought to design a dominant-negative mutant that could be used to remove the three EB family members from MT plus-ends. EBs dimerize through a thermodynamically stable coiled-coil domain in the C-terminal part of the protein (Honnappa et al., 2005; Slep et al., 2005) (Fig. 3A). We first investigated residual endogenous EB1 and EB3 proteins were negligible while the expression of the EB1ΔAc mutant was comparable to the level of endogenous EB1 in control cells (Fig. 2, F and G). Both EB1 and EB1ΔAc expression fully restored persistent MT growth in EB1/EB3-depleted cells (Fig. 2H; Table I). Moreover, we observed no difference in catastrophe frequency after the expression of either full-length EB1 or the EB1ΔAc mutant (Table I). This suggests that EB1 might control persistent MT growth independently of its interaction with the CLIPs, dynactin, CLASPs, APC, and other partners that bind to the EBs in a tail-dependent fashion (Hayashi et al., 2005; Honnappa et al., 2005; Komarova et al., 2005; Mimori-Kiyosue et al., 2005; Weisbrich et al., 2007).

**Table I. Parameters of MT growth in CHO-K1 cells**

| Expression of shRNA | Number of observations | Length of uninterrupted growtha | Growth rateb | Catastrophe frequencyc |
|---------------------|------------------------|---------------------------------|--------------|------------------------|
| Luciferase; YFP-CLIP-170 | 100 MTs; 7 cells | 15.6 ± 8.8 | 24.3 ± 8.3 | 0.3 ± 0.1 |
| Luciferase; Cy3-tubulin | 37 MTs; 3 cells | 13.4 ± 5.9 | 18.5 ± 5.3 | 0.2 ± 0.1 |
| EB1; YFP-CLIP-170 | 88 MTs; 7 cells | 14.5 ± 6.4 | 21.4 ± 9.4 | 0.3 ± 0.1 |
| EB1/EB3; YFP-CLIP-170 | 101 MTs; 8 cells | 7.3 ± 4.5 | 22.6 ± 8.4 | 3.2 ± 0.9 |
| EB1/EB3; Cy3-tubulin | 56 MTs; 3 cells | 7.4 ± 4.1 | 22.4 ± 5.5 | 3.7 ± 0.7 |

**Rescue experiments in EB1/EB3-depleted cells**

| EB1 rescue; YFP-CLIP-170 | 64 MTs; 7 cells | 15.1 ± 7.0 | 21.1 ± 8.0 | 0.3 ± 0.1 |
| EB1ΔAc rescue; YFP-CLIP-170 | 96 MTs; 10 cells | 16.6 ± 7.3 | 26.9 ± 10.7 | 0.3 ± 0.1 |

**Expression of EB1 mutants**

| EB1-NL (control); YFP-CLIP-170 | 88 MTs; 7 cells | 18.5 ± 7.1 | 22.5 ± 7.1 | 0.2 ± 0.1 |
| EB1-CΔAc; YFP-CLIP-170 | 55 MTs; 9 cells | 4.8 ± 3.4 | 18.7 ± 7.8 | 3.5 ± 0.9 |
| EB1-CΔAc; YFP-CLIP-170 | 116 MTs; 4 cells | 4.5 ± 2.9 | 20.3 ± 7.9 | 4.2 ± 1.0 |
| EB1-CΔAc; YFP-CLIP-170 | 55 MTs; 4 cells | 4.8 ± 3.4 | 18.7 ± 7.8 | 3.5 ± 0.9 |

*The length of YFP-CLIP-170 or tubulin tracks represents the length of persistent growth.

*The growth rate ± SD was calculated from the histogram of displacements of YFP-CLIP-170-positive tips or Cy3-labeled MT ends between successive frames.

*The catastrophe frequency (the number of transition events over time) was calculated for each cell and was presented as an average ± SD for the cell population.

**Table II. Parameters of MT shortening in CHO-K1 cells**

| At the cell periphery | In internal cytoplasm |
|-----------------------|-----------------------|
| Number of observations | μm/min | min⁻¹ | μm | Number of observations | μm/min | min⁻¹ | μm |
| Control cells | | | | | | | |
| 200 MTs; 10 cells | 30.0 ± 9.4 | 10.0 ± 1.8 | 2.9 ± 3.4 | 58 MTs; 7 cells | 29.6 ± 11 | 8.1 ± 1.8 | 3.45 ± 2.4 |
| EB1/EB3 depleted cells | | | | | | | |
| 118 MTs; 6 cells | 23.4 ± 10.8 | 10.0 ± 1.5 | 2.4 ± 2.5 | 75 MTs; 5 cells | 23.2 ± 8.8 | 6.7 ± 2.0 | 4.3 ± 3.0 |
| EB1-CΔAc | | | | | | | |
| 102 MTs; 5 cells | 23.7 ± 8.6 | 7.8 ± 2.0 | 2.9 ± 2.3 | | | | |

N/A, not applicable. The analysis was precluded by extremely low catastrophe frequency in internal cell regions.

*The shortening rate ± SD was calculated from the histogram of displacements of shortening MT tips between successive frames using subtracted images.

*The rescue frequency (the number of transition events over time) was calculated for each cell and was presented as an average ± SD for the cell population.

*The length of uninterrupted shortening.

**EB1 dimerization domain acts as a dominant-negative mutant**

Because we could not achieve a complete knockdown of all EB species, we sought to design a dominant-negative mutant that could be used to remove the three EB family members from MT plus-ends. EBs dimerize through a thermodynamically stable coiled-coil domain in the C-terminal part of the protein (Honnappa et al., 2005; Slep et al., 2005) (Fig. 3A). We first investigated
factors, and/or the presence of the linker region in EB1-C and EB1-C/H9004Ac.

Finally, we investigated if endogenous EBs heterodimerize. Using antibodies specific for the three EB species, we found no heterodimer formation with EB2, but a significant co-IP of endogenous EB1 and EB3 (Fig. 3F). This result was not due to cross-reactivity of the used EB3 antibody with EB1 because we observed no co-IP of EB1 with the EB3 antibody from HeLa cells, where EB3 is expressed only at very low levels and is not immunoprecipitated efficiently (unpublished data). A weak co-IP of EB3 with EB1 was also observed; the differences in efficiency of the co-IP of EB1-EB3 dimers with EB1 and EB3 antibodies is most likely caused by the fact that EB1 is expressed at higher levels than EB3 in CHO-K1 cells (unpublished data), and its relative proportion tied up in heterodimers is lower. Based on these data we conclude that EB1 and EB3 can form heterodimers in cells.

Formation of a heterodimer between a full-length EB molecule and the C-terminal fragment will produce a protein that would have only one MT-binding domain and a reduced affinity for MTs (see below). In line with this idea, overexpression of EB1-C and EB1-CΔAc had a profound effect on the distribution of all EB family members, whereas the overexpression of EB1-NL had no consequences for the endogenous EB distribution (Fig. S1, and unpublished data). Quantification whether the EB dimerization domains are kinetically stable by mixing purified C-terminal fragments of EB1, EB2, and EB3 (EB1/2/3c) in various combinations at 37°C and analyzing the mixtures by native PAGE at 4°C. We observed significant and spontaneous heterodimer formation between EB1c and EB3c but only very little or no dimerization with EB2c (Fig. 3B, arrowheads). This result suggests that EBs can potentially heterodimerize. We next investigated if the C-terminal domain of EB1 can disrupt the full-length EB1 and EB3 dimers, and indeed observed heterodimer formation (Fig. 3C, arrowheads). Similar results were obtained when EB3c was mixed with full-length EB1 and EB3 (unpublished data). All analyzed proteins looked homogenous on denaturing and reducing gels (Fig. 3D), indicating that the appearance of additional bands on the native gels resulted from heterodimerization.

Next, we used co-immunoprecipitation (co-IP) to investigate whether heterodimerization can also occur in cells. The C-terminal EB1 fragments EB1-C and EB1-CΔAc (which lacks the acidic tail, Fig. 3A), but not the N-terminal portion of EB1 (EB1-NL, lacking the coiled-coil domain and the C terminus, Fig. 3A) coimmunoprecipitated all three EB family members (Fig. 3E). It is unclear why the interaction between the dimerization domains of EB1 and EB2 was inefficient in vitro but did occur in cells; it could be influenced by temperature and buffer conditions, posttranslational modifications, additional cellular factors, and/or the presence of the linker region in EB1-C and EB1-CΔAc.

Finally, we investigated if endogenous EBs heterodimerize. Using antibodies specific for the three EB species, we found no heterodimer formation with EB2, but a significant co-IP of endogenous EB1 and EB3 (Fig. 3F). This result was not due to cross-reactivity of the used EB3 antibody with EB1 because we observed no co-IP of EB1 with the EB3 antibody from HeLa cells, where EB3 is expressed only at very low levels and is not immunoprecipitated efficiently (unpublished data). A weak co-IP of EB3 with EB1 was also observed; the differences in efficiency of the co-IP of EB1-EB3 dimers with EB1 and EB3 antibodies is most likely caused by the fact that EB1 is expressed at higher levels than EB3 in CHO-K1 cells (unpublished data), and its relative proportion tied up in heterodimers is lower. Based on these data we conclude that EB1 and EB3 can form heterodimers in cells.

Formation of a heterodimer between a full-length EB molecule and the C-terminal fragment will produce a protein that would have only one MT-binding domain and a reduced affinity for MTs (see below). In line with this idea, overexpression of EB1-C and EB1-CΔAc had a profound effect on the distribution of all EB family members, whereas the overexpression of EB1-NL had no consequences for the endogenous EB distribution (Fig. S1, and unpublished data). Quantification
uninterrupted MT growth was similar to that in control cells (Table I) and the distribution of the growing plus-ends along the cell radius was steeply ascending (Fig. 4 E). In contrast, the overexpression of the EB1-C ΔAc severely reduced the length of MT growth episodes (Fig. 4, B and D). Although the rate of MT growth (Fig. 4 C) or parameters of MT shortening (Table II) did not significantly change, the catastrophe frequency was increased similarly to the effect of EB1/EB3 depletion (Table I). MT dynamics alteration led to a dramatic reduction in the proportion of growing plus-ends at the cell periphery (Fig. 4 E). We conclude that mammalian EB1 family members ensure persistent MT growth in cells.

Monomeric EB3 mutant tracks growing MT ends in cells and in vitro

We next analyzed the effect of EB1-C ΔAc expression on MT dynamics. This EB1 fragment is deficient in binding to CLIPs, p150 glued, CLASPs, APC, and MCAK and is therefore not expected to directly influence these +TIPs (Hayashi et al., 2005; Honnappa et al., 2005; Komarova et al., 2005; Mimori-Kiyosue et al., 2005; Weisbrich et al., 2007; unpublished data). MT growth was analyzed using Cy3-labeled tubulin or YFP-Clp-170 in cells overexpressing either EB1-C ΔAc or EB1-NL (Fig. 4, A and B; Table I). The latter protein, when overexpressed at high levels, bound weakly along the whole MT lattice and its overexpression did not affect the growth rate or MT growth persistency (Fig. 4, A, C, and D; Table I). The mean length of uninterrupted MT growth was similar to that in control cells (Table I) and the distribution of the growing plus-ends along the cell radius was steeply ascending (Fig. 4 E). In contrast, the overexpression of the EB1-C ΔAc severely reduced the length of MT growth episodes (Fig. 4, B and D). Although the rate of MT growth (Fig. 4 C) or parameters of MT shortening (Table II) did not significantly change, the catastrophe frequency was increased similarly to the effect of EB1/EB3 depletion (Table I). MT dynamics alteration led to a dramatic reduction in the proportion of growing plus-ends at the cell periphery (Fig. 4 E). We conclude that mammalian EB1 family members ensure persistent MT growth in cells.

Figure 4. Expression of the dominant-negative EB1 mutant disrupts persistent MT growth in CHO-K1 cells. (A and B) Representative time-lapse images of YFP-Clp-170 in cells expressing HA-EB1-NL [control] and HA-EB1-C ΔAc [dominant negative] mutants. Images were acquired every 2 s. Tips of three YFP-Clp-170 comets are indicated over time by an arrow, an arrowhead, and a black-and-white arrowhead. Projection analysis and trajectories of individual YFP-Clp-170 comets are generated in the same way as in Fig. 2 A. Time in seconds is shown in the top right corner. Bar, 10 μm. (C and D) Histograms of the instantaneous MT growth rates and the lengths of YFP-Clp-170 tracks in HA-EB1-NL and HA-EB1-C ΔAc-expressing cells. (E) Distributions of the growing MT plus-ends along the cell radius in EB1-NL and EB1-C ΔAc-expressing cells presented as in Fig. 2 E; error bars represent SD.
MT tips in the absence of dimerizer and was strongly bound to MT ends after dimerizer addition (Slep and Vale, 2007). This result was interpreted as an indication that dimerization is essential for the plus-end tracking behavior. An alternative explanation is that monomeric CH domain-containing EB fragments do not efficiently compete with endogenous EBs for MT tips.

A previous study showed that the N-terminal part of EB1 fused to a regulated dimerization domain was unable to bind to MT tips in the absence of dimerizer and was strongly bound to MT ends after dimerizer addition (Slep and Vale, 2007). This result was interpreted as an indication that dimerization is essential for the plus-end tracking behavior. An alternative explanation is that monomeric CH domain-containing EB fragments do not efficiently compete with endogenous EBs for MT tips.

Figure 5. Monomeric N terminus of EB3 tracks growing MT ends in cells. (A) Live images of CHO-K1 cells transfected with GFP-EB1-CΔAc alone or together with EB3-mRFP. Images were processed by applying Blur filter and Unsharp Mask in Photoshop. Bar, 5 μm. (B–G) Live images of control CHO-K1 cells (B–D) or EB1/EB3-depleted cells (E–G) expressing the indicated constructs. Cells expressing similar low levels of the fusion proteins were selected based on average fluorescence intensity. Bar, 5 μm. (H) Ratio of fluorescence intensities at the growing MT tips and in surrounding cytoplasm (after background subtraction), measured from live cell images obtained as described for panels B–G. 10 cells (45–200 MT tips) were measured for each construct; error bars represent SD. The differences between the indicated values were significantly different; statistical analysis was performed using nonparametric Mann-Whitney U test.
and therefore their accumulation at MT ends is low. If this assumption were correct, the binding of monomeric CH domain–
containing EB fragments to MT tips should improve in the absence of endogenous EBs. To test this idea, we first confirmed that the EB3 N-terminal portion, artificially dimerized by the addition of the leucine zipper (LZ) domain of GCN4 (EB3-NL-LZ), efficiently accumulated at MT tips, indicating that the coiled-coil domain and the acidic tail of EB3 are not needed for plus-end tracking (Fig. 5, B and H; see Fig. 7 for a scheme of the fusions). An artificial dimer of the EB3 CH domain lacking most of the linker region (EB3-N-LZ) also tracked plus-ends, albeit less efficiently (Fig. 5, C and H), indicating that the linker region of the EBs contributes to MT binding but is not essential.

Next, we fused EB3-N to the homodimerization domain FKBP; as a monomer, it showed hardly any plus-end tracking in control cells (unpublished data) but displayed MT end accumulation, albeit weak, in cells depleted of EB1 and EB3 (Fig. 5 E). Plus-end accumulation of this protein was enhanced by dimerizer addition (Fig. 5, F and H), indicating that the monomeric version of the construct can still plus-end track, but dimerization enhances its binding to MT tips.

To exclude potential weak dimerization of EB3-N through FKBP or GFP, we next tested whether EB3-N or EB3-NL fused to a monomeric version of the fluorescent protein Venus (mVenus) can bind MT tips. EB3-NL-mVenus displayed clear plus-end tracking in EB1/EB3 knockdown cells but only a weak plus-end binding in control cells (Fig. 5, D, G, and H). EB3-N-mVenus showed no plus-end accumulation (unpublished data), indicating that the linker region contributes to MT binding, in line with the recently published data on Mal3 (des Georges et al., 2008).

To investigate the oligomerization state of EB3-NL-mVenus, we fused it to a 6xHIS tag and purified it from bacteria. Size-exclusion chromatography and static light scattering (SLS) analysis yielded an average molecular mass of 56.3 kD (Fig. S3 A, available at http://www.jcb.org/cgi/content/full/jcb.200807179/DC1), in agreement with the calculated molecular mass of the protein (51.4 kD). This result shows that EB3-NL-mVenus is indeed a monomer. In contrast, GFP-EB3 displayed an average molecular mass of 128 kD (Fig. S3 A), consistent with the formation of a dimer (calculated molecular mass 127.8 kD).

We next used purified full-length GFP-EB3 and EB3-NL-mVenus fusions to reconstitute plus-end tracking in vitro using the approach described by Bieling et al. (2007). Both the full-length EB3 dimer and the monomeric EB3-NL-mVenus protein weakly decorated the MT lattice and strongly accumulated at the growing plus- and minus-ends; the labeling of the rapidly growing plus-ends was higher than that of slowly growing minus-ends (Fig. 6, A–D; Videos 3 and 4, available at http://www.jcb.org/cgi/content/full/jcb.200807179/DC1). Collectively, our results show that a single CH domain is capable of recognizing growing MT ends in the absence of other +TIPs, and that this process does not rely on dimer formation.

To show that monomeric EB3 binds MT tips less efficiently compared with the dimer, we performed in vitro competition experiments. Increasing levels of purified mCherry-tagged or untagged full-length EB3 were able to suppress the accumulation of EB3-NL-mVenus at the growing MT tips, while its MT lattice binding did not significantly change (Fig. 6, E and F). In contrast, comparable levels of monomeric EB3-NL-mVenus had no effect on the accumulation of mCherry-EB3 at the tips of MTs (Fig. 6, E and F), indicating that the dimeric full-length protein has a higher affinity for the growing MT ends.

**Dimerization is required for the anti-catastrophe activity of EB3 in cells**

We next set out to test which EB domains are important for MT catastrophe suppression. To be able to screen a broad panel of mutants, we switched to transient cotransfection of shRNA and rescue constructs, which contained a fluorescent tag and therefore could be directly used to detect MT growth (Fig. 7). As a measure of persistent growth, we calculated the percentage of tracks longer than 7.5 μm (approximately half of the cell radius) (Fig. 7).

We measured this parameter in control cells by using direct MT labeling with mCherry–α-tubulin and visualizing MT outgrowth from the centrosome within a photobleached cell sector (Fig. S2 E), and by labeling growing MT ends either with GFP-CLIP-170 or EB3-GFP. All three methods produced consistent results and although we used transfection instead of microinjection, MT growth parameters were quite similar to those described above (Fig. 7; Figs. S4 and S5, available at http://www.jcb.org/cgi/content/full/jcb.200807179/DC1). Also under these conditions we observed a strong reduction of persistent MT growth when EB1 and EB3 were depleted, while MT growth rate was not significantly affected (Figs. S4 and S5). In control cells the percentage of tracks longer than 7.5 μm exceeded 80%; it was reduced to ~40% after simultaneous EB1/EB3 depletion (Fig. 7; Fig. S4). The results obtained with GFP-tagged CLIP-170 and mCherry–α-tubulin were again very similar (Fig. 7). Further, by cotransfecting mCherry–α-tubulin with hemagglutinin (HA)-tagged EB1-CΔAc and monitoring MT growth in photobleached regions, we confirmed the severe disruption of MT growth persistence by this dominant-negative mutant of EB1 (Fig. 7).

Persistent MT growth was completely restored by the expression of the full-length EB3 with a C-terminal GFP tag, or by the GFP-EB3-ΔAc fusion, lacking the acidic tail (Fig. 7; Fig. S4). These results are fully consistent with those obtained with untagged EB1 constructs (Fig. 2 H; Table 1), and indicate that the N- or C-terminal GFP tag does not interfere with the catastrophe-suppressing activity of EB3. In contrast, the monomeric protein EB3-NL-mVenus displayed no rescue activity, indicating that accumulation of a single EB3 CH domain at MT ends is by itself insufficient for catastrophe inhibition.

We next tested the artificial EB3 dimers in which the EB3-N or EB3-NL fragments were fused to the GCN4 leucine zipper or FKBP. Interestingly EB3-NL-LZ (but not EB3-N-LZ or the EB3-N-FKBP fusion, with or without dimerizer addition) displayed a significant anti-catastrophe activity: the proportion of long MT growth tracks was increased to ~75% (Fig. 7; Fig. S4). Because EB3-NL-LZ is completely devoid of any sequences responsible for the binding to the known EB partners, its anti-catastrophe activity is most likely due to its effect on the MT tip. We conclude that in cells anti-catastrophe activity of EB3 requires dimerization of the CH domain and is sensitive to the
significantl compromised the catastrophe-inhibiting activity of EB3 (Fig. 7; Fig. S4).

To get insight into the differences between the CH domains of EB3, EB2, and EB1 (see Fig. 8A for sequences), we solved the x-ray crystal structure of EB3-CH domain at 1.4 Å resolution (Table S1). The structure of the CH domain of EB3 is very similar to the ones of EB1 (Hayashi and Ikura, 2003; Slep and Vale, 2007) and its yeast orthologue Bim1 (Slep and Vale, 2007; Fig. 8B). It is formed by seven helices that pack around the central and conserved helix H9251. We failed to obtain crystals of EB2-CH; however, because of the high sequence identity between the CH domains of EB1 and EB2 we modeled the EB2-CH structure (see Materials and methods). Using the structures of the CH domains of EB1 and EB3, together with the structural model of EB2 we analyzed and mapped surface residue differences between the three EBs (Fig. 8, C and D). Based on the data from Slep and Vale (2007), we also mapped conserved residues that abrogated MT plus-end tracking of EB1 in cells (Fig. 8, C and D, blue and adjacent linker sequences, suggesting that the features that increase MT tip association of the EBs are beneficial for catastrophe suppression.

**Differences between the N-terminal parts of the EB2 and EB3 CH domains have a large impact on their catastrophe suppression activity**

The presence of EB2 alone was insufficient to support processive MT growth (Fig. 2B); also an overexpression of the GFP-tagged or untagged EB2 could not rescue the effect of EB1/EB3 depletion (Fig. 7; Fig. S4; and unpublished data). EB2 differs from EB1 and EB3 by the presence of an ~40 amino acid N-terminal extension. We fused this extension to EB3-GFP and found that it had no effect on the capacity of the protein to suppress catastrophes. In contrast, substitution of the first 29 amino acids of EB3 for the corresponding residues of EB2 in the presence or absence of the EB2-specific N-terminal extension significantly compromised the catastrophe-inhibiting activity of EB3 (Fig. 7; Fig. S4).

Figure 6. Monomeric N terminus of EB3 tracks growing MT ends in vitro. (A–D) In vitro MT plus-end tracking assay. Representative TIRFM images (A and C) and kymographs (B and D) show specific accumulation of GFP-EB3 (100 nM) and EB3-NL-mVenus (100 nM) at the growing, but not shortening MT plus (+) and minus (−) ends. Horizontal bars, 5 μm; vertical bar, 60 s. (E) Dual-color imaging of in vitro plus-end tracking assays performed with the indicated concentrations of EB3-NL-mVenus and mCherry-EB3. Images were processed by applying Blur filter in Photoshop. Bar, 1 μm. (F) Ratio of fluorescence intensity at the growing MT tip and on the MT lattice for the indicated protein mixtures (after background subtraction). Green plots show measurements for EB3-NL-mVenus and red plots for mCherry-EB3. 10–20 MT tips were measured per experiment; error bars represent SD.
Interestingly, the monomeric EB3-NL-mVenus had a very similar, albeit a weaker effect, compared with the full-length protein (Fig. S3 B, Table III), indicating that, at least in conditions used by us in vitro, EB3 does not act through a dimerization-dependent tubulin multimerization, as has been proposed previously (Slep and Vale, 2007). Further, these results show that catastrophe suppression observed in cells is most likely not an intrinsic activity of EB3.

### Discussion

The EBs are highly conserved proteins, which can target growing MT tips on their own and are involved in the recruitment of numerous other +TIPs to MT ends, suggesting that they hold the key to understanding the plus-end tracking phenomenon. Using in vitro and in vivo approaches we have shown that a single EB CH domain is necessary and sufficient for specific association with growing MT ends. The linker region, adjacent to the CH domain, as well as the C-terminal dimerization domain, can contribute to MT tip accumulation but are not essential because EB3-N-FKBP and EB3-N-LZ, which lack these domains, still track MT plus-ends. This indicates that a single CH domain is the primary determinant of MT plus-end recognition.

Although dimerization is not required for plus-end tracking, EBs do exist as dimers (Honnappa et al., 2005; Slep et al., purple). Interestingly, most of the residue differences found at the N terminus of EB3-CH domain and EB2-CH domain cluster around a patch of residues formed by the conserved sequence segment 16-SRHD-19, which is critical for MT binding (Fig. 8 C, red). In contrast, differences between EB3 and EB1 are less pronounced: the few residue substitutions are mostly localized in regions that do not affect MT end accumulation (Fig. 8, C and D, green and orange). These findings help to explain why EB3 and EB1 display similar MT binding and catastrophe suppression behaviors, and why EB2 activity is distinct.

**EB3 promotes MT catastrophes in vitro**

To address whether anti-catastrophe activity is an intrinsic property of EBs, we analyzed the impact of the full-length and monomeric EB3 on MT dynamics reconstituted with purified tubulin in vitro, by imaging MTs with differential interference contrast (DIC) microscopy. The full-length EB3 (with or without the GFP tag) increased MT growth velocity and the frequency of catastrophes and rescues (Fig. S3 B, Table III). This is in line with some previous work on EB1 and Mal3 (Bieling et al., 2007; Vitre et al., 2008), although little effect on MT dynamics in vitro was found in another study (Dixit et al., 2009), and catastrophe suppression by EB1 was described in yet another paper (Manna et al., 2007). These differences are possibly due to use of different tubulin preparations and assay conditions.

| Scheme | Number of long MT dynamics (% >7.5 μm) | Amino acids | Scheme | Number tracks/cells |
|--------|--------------------------------------|-------------|--------|---------------------|
| Control cells |  | EB2-EB3 chimeras | Rescue with EB3 fusions |  |
| mCherry-tubulin | dimer | 1-281 | CHD L CC Ac | GFP |
| GFP-CLIP-170 | dimer | 1-258 | CHD L CC Ac | GFP |
| EB3-NL-mVenus | dimer | 1-137 | FKBP GFP | GFP |
| EB3-N-FKBP-GFP | dimer | 1-137 | FKBP GFP | GFP |
| EB3-N-LZ-GFP | dimer | 1-200 | LZ GFP | GFP |
| EB3-NL-LZ-GFP | dimer | 1-326 |  |  |

**Figure 7. Rescue of processive MT growth by different EB3 fusions and EB2-EB3 chimeras in EB1/EB3-depleted cells.** The proportion of MT tracks originating from the centrosome, with the length exceeding 7.5 μm, schematic representations of the constructs, amino acid positions in EB3 and EB2, and the numbers of tracks and cells analyzed for each construct are indicated. Share of long MT growth episodes in control cells, EB1/EB3-depleted cells, and cells expressing HA-EB1-A cAc obtained using GFP-CLIP-170 or mCherry-tubulin (after photobleaching) is shown for comparison. Cells with approximately the same radius were selected for quantification. Error bars indicate SD determined based on 2–4 independent experiments.
Unexpectedly, these dimers readily exchange their subunits in cells and as purified proteins in vitro. This property seems to be more pronounced for EB1 and EB3, compared with EB2. We show that heterodimerization between EB1 and EB3 occurs at endogenous expression levels; it might increase the structural and functional diversity of the EBs. Subunit exchange between EB dimers also explains why overexpression of the C-terminal EB1 fragment has a dominant-negative effect on the endogenous EBs. Until now, overexpression of the EB1 or EB3 C termini was mainly used as a tool aimed at hindering the interaction between the EBs and their binding partners (Askham et al., 2002; Wen et al., 2004; Zhou et al., 2004; Etienne-Manneville et al., 2005; Geraldo et al., 2008). Our findings indicate that the data obtained by overexpression of these EB fragments must be interpreted with caution because EBs themselves are their primary targets.

By using a combination of approaches we show that in cells EBs (in particular EB1 and EB3) have little effect on MT growth rate or rescues, but suppress catastrophes. This is in line with observations in fission yeast and in Xenopus extracts, where EB homologues displayed an anti-catastrophe activity (Tirnauer et al., 2002; Busch and Brunner, 2004). In contrast, in our hands EB3 increases MT growth velocity and promotes both catastrophes and rescues when MT dynamics is reconstituted with purified proteins. An important conclusion of our study is that this change can be brought about by a monomer of a CH domain with an adjacent linker sequence. We favor the idea that EB accumulation changes the structure of the MT tip (des Georges et al., 2008; Vitre et al., 2008), and thus promotes MT growth but also increases the probability of a catastrophe in vitro.

To explain the discrepancy between the in vitro and in vivo results, we propose that the activities measured in these conditions are fundamentally different. This is supported by the fact that the structural requirements for the observed effects are different as well: in cells, only EB3 mutants that form dimers inhibit catastrophes, whereas in a purified system a considerable effect on MT dynamics is produced by monomeric EB3. What we measure in cells is most likely not an intrinsic activity of the EBs, but rather their effect on activities of other MT-regulating factors.

Because the EBs bind to multiple +TIPS, it was important to determine if their catastrophe-suppressing activity depends on direct interactions with any of these factors. Two lines of evidence suggest that this is not the case. First, we have shown that EB1 or EB3 lacking the C-terminal acidic tail essential for interactions with the major EB partners are sufficient to completely restore progressive MT growth in EB1/EB3-deficient cells. Second, an artificial EB3 dimer, which lacked the whole
C-terminal partner-binding domain, displayed a significant catastrophe-inhibiting activity.

On the other hand, catastrophe suppression by the EBs in cells is strongly correlated with their capacity to bind MT tips because dimerization and the presence of the linker region have a positive effect on both properties. Furthermore, EB2, which binds MT tips less prominently compared with EB1 and EB3, is also a much less potent inhibitor of catastrophes. In this respect, EB2 behaves similar to monomeric EB3-NL, which is also unable to support processive MT growth or efficiently compete with full-length EB1 and EB3 for the binding sites at MT ends. The relevant differences between EB2 and EB1/EB3 may indeed influence MT binding because they cluster around CH domain residues important for MT plus-end accumulation (Fig. 8, C and D). These results suggest that the EBs suppress catastrophes through an interaction with MTs. The EBs could stabilize contacts between protofilaments or the lattice seam, as previously suggested (Sandblad et al., 2006; Vitre et al., 2008). They could thus counteract the function of MT-destabilizing factors either directly, by occluding binding sites on the MTs, or indirectly, by altering the MT tip structure.

Although in an in vitro reconstitution system the EBs also influence MT growth rate and the frequency of rescues, this seems not to be the case in cells. It should be noted that in our experiments we were unable to achieve a complete depletion of all three EBs; therefore, we cannot exclude that a more complete knockdown would have an effect on these parameters. Still, it is noteworthy that although EB1 and EB3 directly bind to several important MT rescue factors, such as the CLIPs (Komarova et al., 2002a, 2005), they have very little effect on MT rescue. On the other hand, loss of the CLIPs from MT plus-ends does not affect catastrophes (Komarova et al., 2002a). It appears, therefore, that in spite of their direct interaction, the CLIPs and the EBs act on MTs independently, similar to the situation in fission yeast (Wolnyiak et al., 2006). The seemingly independent actions of the EBs and the CLIPs ensure low MT catastrophe and high rescue frequencies, thus resulting in persistent MT growth in the cell interior and a strong accumulation of dynamic MT ends near the cell margin. The existence of long and continuous MT tracks facilitates the traffic between the cell center and cell periphery; it also favors the interactions of dynamic MT ends with the cell cortex that is essential for cell polarization and migration.

### Materials and methods

#### Protein and shRNA expression constructs, co-IP, and Western blotting

Human EB3-GFP was described by Stepanova et al. (2003); pSuper-based vectors to knock down EB1, EB2, EB3, and the control vector (expressing luciferase-specific shRNA), EB1 rescue constructs, and the EB2 expression construct (based on mouse cDNA) were described by Komarova et al. (2005). HA-tagged EB1 deletion mutants were generated by a PCR-based strategy and subcloned into the pEGFP-N1 vector (Clontech Laboratories, Inc.), which was modified by substituting the GFP open reading frame by a triple HA tag. Homodimerization domain FKBP and the rapamycin-derived homodimerizer AP20187 were obtained from Ariad (http://www.ariad.com/wr/page/regulation_kits). Different EB3 constructs and EB2-EB3 chimeras were generated by PCR and cloned into pEGFP-N1 or pEGFP-C1. GFP-EB3ΔAc was generated in pEGFP-C1 with a linker sequence AAGGSGGSGGSGGSDG inserted between GFP and EB3. EB3-NL-mVenus was cloned into pEGFP-N1 from which the GFP open reading frame was removed; mVenus was generated by introducing the A206K mutation (Zacharias et al., 2002) into the Venus-encoding plasmid (a gift of A. Miyawaki; RIKEN, Wako City, Japan). mCherry-EB3 was derived from pEGFP-N1 by introducing the target sequence AQAGGSGGAGSGGEGAVDG inserted between GFP and EB3.

#### Construction of the bacterial EB1c expression vector

Construction of the bacterial EB1c expression vector is described by Honnappa et al. (2005). Human EB1, EB2c, EB3, and EB3c were PCR-amplified from human EB1, EB2, and EB3 full-length cDNA clones (provided by W. Bu; University of Texas, Houston, TX). Using the Gateway cloning system (Invitrogen) all PCR products were subcloned into a pDONR221 vector by BP reactions and thereafter cloned by LR reactions into a pDEST17 vector according to the manufacturer’s instruction. Proteins were expressed in *Escherichia coli* [B121 (DE3) and purified on HiTrap Ni²⁺-Sepharose chelating columns (GE Healthcare) as described previously (Honnappa et al., 2005). After cleavage of the 6xHis-tag by thrombin at 4°C overnight, the proteins were gel filtrated on a Superdex-200 column (GE Healthcare) equilibrated in 20 mM Tris-HCl, pH 7.4, 0.15 M NaCl, and 2 mM dithiothreitol. GFP-EB3 and mCherry-EB3 containing the same linker as GFP-EB3ΔAc and EB3-NL-mVenus were subcloned into pET28a and purified as described above; the 6xHis-tag was not removed.

#### Cloning, protein preparation, and SLS analysis

Construction of the bacterial EB1c expression vector is described by Honnappa et al. (2005). Human EB1, EB2c, EB3, and EB3c were PCR-amplified from human EB1, EB2, and EB3 full-length cDNA clones (provided by W. Bu; University of Texas, Houston, TX). Using the Gateway cloning system (Invitrogen) all PCR products were subcloned into a pDONR221 vector by BP reactions and thereafter cloned by LR reactions into a pDEST17 vector according to the manufacturer’s instruction. Proteins were expressed in *Escherichia coli* [B121 (DE3) and purified on HiTrap Ni²⁺-Sepharose chelating columns (GE Healthcare) as described previously (Honnappa et al., 2005). After cleavage of the 6xHis-tag by thrombin at 4°C overnight, the proteins were gel filtrated on a Superdex-200 column (GE Healthcare) equilibrated in 20 mM Tris-HCl, pH 7.4, 0.15 M NaCl, and 2 mM dithiothreitol. GFP-EB3 and mCherry-EB3 containing the same linker as GFP-EB3ΔAc and EB3-NL-mVenus were subcloned into pET28a and purified as described above; the 6xHis-tag was not removed.

#### Table III. Parameters of MT dynamics in vitro

|                     | Growth rate  | Shortening rate | Catastrophe frequency | Rescue frequency |
|---------------------|--------------|-----------------|-----------------------|-----------------|
|                     | μm/min       | μm/min          | min⁻¹                 | min⁻¹           |
| Control             | 0.50 ± 0.19  | 10.37 ± 2.85    | 0.13                  | 0.09            |
| n = 107             | n = 99       | n = 125         | n = 136               | n = 2           |
| EB3                 | 2.36 ± 0.48  | 15.50 ± 4.85    | 1.17                  | 0.59            |
| [200 nM]            | n = 126      | n = 125         | n = 136               | n = 11          |
| EB3                 | 2.56 ± 0.43  | 14.18 ± 2.59    | 1.00                  | 0.26            |
| [1 μM]              | n = 185      | n = 185         | n = 185               | n = 9           |
| EB3-GFP             | 2.75 ± 0.37  | 14.67 ± 2.46    | 0.76                  | 0.40            |
| [200 nM]            | n = 85       | n = 85          | n = 85                | n = 9           |
| EB3-GFP             | 1.93 ± 0.28  | 13.57 ± 2.11    | 0.97                  | 0.40            |
| [1 μM]              | n = 33       | n = 33          | n = 33                | n = 2           |
| EB3-NL-mVenus       | 1.82 ± 0.29  | 11.86 ± 2.43    | 0.84                  | 0.54            |
| [200 nM]            | n = 84       | n = 84          | n = 84                | n = 9           |
| EB3-NL-mVenus       | 1.58 ± 0.27  | 10.53 ± 1.83    | 0.76                  | 0.16            |
| [1 μM]              | n = 88       | n = 88          | n = 88                | n = 3           |

The number of events measured for each condition (n) is indicated below each measurement. Growing and shortening rates were determined from kymograph slopes corresponding to individual growth or shrinkage phases. An average rate was determined as an average over all events weighted with the time of individual events (as weighted SD). Transition frequencies were determined as a number of events divided by time spent in the growing or shrinking phase, respectively.
EB3 CH domain (1.7 mM; residues 1–130) was crystallized at 20 °C by Crystal structure determination and modeling and 9% native PAGE gels, respectively. Run on an Agilent 1100 HPLC. 100 μl of with an Optilab rEX refractometer (Wyatt Technology corp.), which was failed to crystallize the EB3 CH domain. However, because of the high sequence conservation between the CH domains of EB2 and EB1 (sequence identity and sequence similarity of 77% and 91%, respectively) we modeled the structure of EB2 CH (residues 44–173) using the SWISS-MODEL software package (Arnold et al., 2006) and using the EB1 CH structure (PDF ID 1PA7) as a template. Structural figures were prepared with the program PyMOL (DeLano Scientific, LLC).

Cell culture, transfection, and microinjection

The CHO-K1 cell line stably expressing YFP-CLIP-170 was described previously (Komarova et al., 2005). To introduce different expression vectors into cells we used microinjection or transient transfection. Cells were plated on coverslips in culture dishes with phototetched glass coverslips (Belloco Glass) and 12–24 h later they were microinjected with different shRNA-expressing plasmids into nuclei. The needle concentration was 100 μl/g DNA for both plasmids and 70 μg/ml for EB expression constructs in the rescue experiments with EB1, EB2, or EB1ΔAc. To observe MT behavior directly we used a CFP-expressing EB1/EB3 shRNA construct (Komarova et al., 2005); CFP-positive cells were microinjected into the cytoplasm with Cy3-labeled tubulin at a needle concentration of 10 mg/ml. Cells were fixed or used for live observation 72–90 h after microinjection. In experiments where cotransfection of shRNA and rescue constructs was used, cells were transfected with FuGene 6 (Roche) at 50% confluence, replicated at low density on coverslips 24 h later, and analyzed by live imaging after an additional 72 h in culture.

Antibodies

We used rat monoclonal antibodies KT51, KT52, and KT53 against EB1, EB2, and EB3 proteins (Absea); mouse monoclonal antibodies EB1 and EB3 (BD Biosciences), HA tag (Babco), a rat monoclonal antibody against α-tubulin (YLI/2, Abcam), and rabbit antibodies against EB3 (D2–1005-07, Stepanova et al., 2003) and GFP (Abcam and Invitrogen). The following secondary antibodies were used: alkaline phosphatase–conjugated anti-rabbit and anti–mouse antibodies (Sigma–Aldrich), TRITC–FITC–conjugated donkey anti–mouse and anti–rabbit and Cy5–conjugated anti–rat antibodies (Jackson ImmunoResearch Laboratories).

Immunostaining and analysis of EB protein accumulation at the MT tips

Cell fixation and staining were performed as described previously (Komarova et al., 2002a). Fixed samples were analyzed by fluorescence deconvolution microscopy using a DeltaVision microscope system equipped with an Olympus IX70 inverted microscope and a PlanApo 60x 1.4 NA objective. We used x2 binning that gave a resolution of 0.22 μm/pixel. Images were prepared for presentation using Adobe Photoshop. Analysis of fluorescence intensity distribution of EBs along MTs was performed using MetaMorph and SigmaPlot software as described by Komarova et al. (2005). In brief, the intensity profiles from different microtubule ends were aligned based on the strong increase in pixel intensity compared with the background (the point where pixel intensity abruptly changed was considered as the microtubule tip). Because line-scan analysis was performed on the combined three-color images (obtained using DeltaVision software) and there was no obvious chromatic shift, the position of the microtubule tip was determined based on the profiles of the expressed EB family members in the case when other members were depleted.

Live imaging, image processing, and quantification of MT dynamics

Microinjected CHO-K1 cells were imaged on the Nikon Diaphot 300 inverted microscope equipped with a Plan 100x, 1.25 N.A. objective and YFP, GFP, and Cy3 filter sets. Cells injected with Cy3-tubulin were treated with 0.1% Triton X-100 for 3 min after microinjection, rinsed with assay buffer supplemented with 50 mM KCl, 0.1% Triton X-100, and 200 μM MgCl2 and then fixed with 1% formaldehyde in assay buffer supplemented with 30 mM KCl. 100 μM EB protein solutions were mixed in different ratios and incubated for 5 min at 37°C. EB heterodimers and EBC/EB full-length heterodimers were analyzed at 4°C on 15% and 9% native PAGE gels, respectively.

For transfection–based experiments, cells were imaged on the inverted Nikon Eclipse TE2000E microscope equipped with a CFI Apo TIRF 100x 1.49 N.A. oil objective (Nikon), equipped with a QuantiEM EMCCD camera (Roper Scientific) controlled by MetaMorph software. For excitation we used an HBO 103 W/2 Mercury Short Arc Lamp (Osram) and a Chroma ET GFP filter cube. Images were collected with ~0.5 s exposure in a stream mode.

Total internal reflection fluorescence microscopy (TIRFM) imaging for the in vitro plus-end tracking assay was performed on the same setup, using the 113-nm, 488-nm laser line of an argon laser (Spectra-Physics Lasers) and 11-nm, 561-nm diode-pumped solid-state laser (Melles Griot) for excitation. For separation of emissions we used DualView (Optical Insight) with emitters HG530/30M and HG630/50M (Chroma Technology Corp.) and the beam splitter 565DCXR (Chroma Technology Corp.). The 16-bit images were processed using MetaMorph and SigmaPlot software as described elsewhere (Komarova et al., 2002a).

Images were prepared for presentation with Adobe Photoshop by cropping and adjusting contrast and levels. Additional processing is indicated in the figure legends.

In vitro plus-end tracking assay

Plus-end tracking assay was performed as described by Bielei et al. (2007), with some modifications. Flow chambers were assembled between a pre-cleaned glass coverslip and a microscope slide that were glued together using Dow Corning high vacuum silicone grease (Sigma–Aldrich). The chamber was incubated with 0.2 mg/ml PLL-PEG-biotin (Susos AG) in assay buffer (80 mM K-PIPES, pH 6.8, 4 mM MgCl2, and 1 mM EGTA) and after rinsing with 1 mg/ml casein. Accumulation of GFP-tagged +TIPs at the ends of dynein–casein, 0.1% methylcellulose (4000cp) (Sigma–Aldrich), and an oxygen scavenger system (100 mM glu- case, 400 μg/ml glucose–oxidase, 200 μg/ml catalase, and 4 mM DTT) were prepared for presentation using Adobe Photoshop by cropping and adjusting contrast and levels. During experiments the samples were kept at 26 ± 1°C; images were collected using TIRFM.

Measurement of MT dynamics in vitro using DIC microscopy

MTs were nucleated from seeds (preparated as described above, but without rhodamine–tubulin) bound to the surface of a flow chamber as described above. MT growth was initiated by flowing in a solution containing 15 μM tubulin, 1 mM GTP, and EB3 constructs (at a concentration of 0.2 or 1 μM) in assay buffer supplemented with 50 mM KCl, 0.1% methyl cellulose, 0.3 mg/ml casein, and an oxygen scavenger system. MTs were imaged by video-enhanced DIC microscopy, using an inverted microscope equipped with a CFI Apo TIRF 100x 1.49 N.A. oil immersion objective (HCX PL-
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