EB1 Accelerates Two Conformational Transitions Important for Microtubule Maturation and Dynamics

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Summary

Background: The dynamic properties of microtubules depend on complex nanoscale structural rearrangements in their end regions. Members of the EB1 and XMAP215 protein families interact autonomously with microtubule ends. EB1 recruits several other proteins to growing microtubule ends and has seemingly antagonistic effects on microtubule dynamics: it induces catastrophes, and it increases growth velocity, as does the polymerase XMAP215.

Results: Using a combination of in vitro reconstitution, time-lapse fluorescence microscopy, and subpixel-precision image analysis and convolved model fitting, we have studied the effects of EB1 on conformational transitions in growing microtubule ends and on the time course of catastrophes. EB1 density distributions at growing microtubule ends reveal two consecutive conformational transitions in the microtubule end region, which have growth-velocity-independent kinetics. EB1 binds to the microtubule after the first and before the second conformational transition has occurred, positioning it several tens of nanometers behind XMAP215, which binds to the extreme microtubule end. EB1 binding accelerates conformational maturation in the microtubule, most likely by promoting lateral protofilament interactions and by accelerating reactions of the guanosine triphosphate (GTP) hydrolysis cycle. The microtubule maturation time is directly linked to the duration of a growth pause just before microtubule depolymerization, indicating an important role of the maturation time for the control of dynamic instability.

Conclusions: These activities establish EB1 as a microtubule maturation factor and provide a mechanistic explanation for its effects on microtubule growth and catastrophe frequency, which cause microtubules to be more dynamic.

Introduction

The microtubule cytoskeleton is essential for intracellular organization, transport, and division of eukaryotic cells. Microtubules are structurally polar and dynamic filaments that grow by addition of guanosine triphosphate (GTP)-loaded tubulin subunits to their end. After complex, largely unknown structural rearrangements at the nanoscale, GTP hydrolysis and phosphate release lead to the formation of a guanosine diphosphate (GDP)-loaded microtubule lattice. The matured microtubule lattice is protected from depolymerization by a stabilizing structure at the growing microtubule end. Stochastic loss of this end structure leads to depolymerization (catastrophe) [1]. The dynamic properties of microtubules are regulated by multiple proteins. Two of them, EB1 and XMAP215 (chTOG in humans), are special in that they accumulate autonomously at microtubule ends [2, 3]. EB1 is selective for growing ends (not distinguishing between plus and minus ends) [4–10], whereas XMAP215 is selective for plus ends (not distinguishing between growing and shrinking ends) [3, 11], suggesting different binding modes.

Members of the EB1 family (EBs) are mostly known for recruiting a variety of other plus-end-tracking proteins through interactions with their C-terminal EB homology domain [7, 12–14]. The N-terminal microtubule binding domains bind to the outer microtubule surface in the grooves between adjacent protofilaments, close to the exchangeable GTP binding site [15]. In addition to recognizing these binding sites, EB1 senses conformational changes within the microtubule lattice induced by reactions taking place as part of the GTP hydrolysis cycle [15, 16]. This leads to the well-known comet-like accumulation of EBs at the end region of growing microtubules where high-affinity binding sites are gradually lost with time [4]. Varied effects on microtubule dynamics have been reported for EB family members, with most experiments suggesting a promotion of the microtubule growth rate and an increase of the catastrophe frequency [4, 17–21], seemingly antagonistic activities.

XMAP215 binds to the microtubule lattice and with its TOG domains also to free tubulin [22], which is the basis for its capacity to catalyze tubulin exchange at microtubule plus ends. Under conditions allowing microtubule growth, XMAP215 acts as a processive polymerase, but it can also catalyze depolymerization [3, 23]. The interaction mode of XMAP215 with soluble tubulin is known from a recent X-ray structure [24], but the precise binding site on the microtubule surface is unclear. EB1 enhances the polymerase activity of XMAP215 in vitro [19], although EB1 and chTOG have different and independent microtubule binding sites, as suggested by competition experiments in living cells [25]. Structured illumination microscopy of fixed cells showed a shifted peak of the fluorescence intensity profile of labeled EB1 along microtubule ends with respect to the chTOG signal peak position [25]; however, convolution effects of the optical microscope were not considered, and hence the nanoscale density distributions of EB1 and XMAP215 at microtubule ends remain unknown. Differences in these distributions could provide valuable information about structurally different zones at growing microtubule ends, and hence about how growing microtubule ends develop into mature tubes.

Here, we have performed simultaneous dual-color time-lapse total internal reflection fluorescence (TIRF) microscopy of dynamic microtubules in the presence of EB1 or XMAP215 in vitro. Using automated image data analysis with subpixel
precision and convolved model fitting, we extracted nano-
scale molecular density distributions for EB1 and XMAP215
at growing microtubule ends from their measured fluores-
cence intensity profiles. We find that growing microtubules
mature in at least two distinct kinetic steps and that both
conformational transitions are accelerated by EB1. Speeding
up microtubule maturation, probably by promoting lateral
protofilament contacts, and increasing the rate of GTP hy-
drolysis and/or phosphate release shortens the lifetime of the
microtubule’s protective end structure, which explains the
catastrophe-promoting activity of EB1. Our findings provide
new insights into conformational rearrangements at the
nanoscale taking place when microtubules grow, and into
how modulation of these rearrangements regulates micro-
tubule dynamics.

Results

Molecular Density Distributions from Averaged
Fluorescence Intensity Profiles

To gain insight into how EB1 could influence microtubule
growth and catastrophe, we examined the fluorescence pro-
files of growing microtubule end regions using dual-color
TIRF microscopy with subpixel precision. We imaged purified
GFP-tagged human EB1 or, for comparison, XMAP215-GFP
and after convolution); and smoothens out step functions
(as expected in the simplest case for the microtubule end;

Figure 1B, red). Therefore, we fitted the experimentally deter-
mined superaveraged one-dimensional intensity profiles with
mathematical functions produced by convolving an effective
PSF (a Gaussian function) with models for the underlying mo-
lecular density distributions (see Supplemental Experimental
Procedures).

Molecular Density Distributions of EB1 Shift Away from
Microtubule Ends with Increasing Growth Velocity

We first analyzed the fluorescence intensity profiles of Cy5-
microtubules growing at three different growth velocities in
the range from 49 to 95 nm/s in the presence of purified
EB1-GFP (Figures 2A, S2A, and S2B). Averaging allowed us
to generate fluorescence intensity profiles with a high signal-
to-noise ratio at very low EB1-GFP concentrations of 1 nM.
This revealed a shift of the EB1-GFP intensity peak away
from the microtubule end, which increased with increasing
microtubule growth velocity (Figure 2C).

No changes were observed in the Cy5-microtubule end pro-
files, which could be well fitted using an error function, i.e.,
a convolution of a step function with a Gaussian function
representing the effective PSF (Figures 2B and 2E). The value
(see Figure 1B and Supplemental Experimental Procedures)
of the Gaussian PSF did not change significantly with the micro-
tubule growth velocity: even at the highest velocity, it was
within 10 nm of our optical PSF of 135 nm, which we also found
when measuring the ends of blunt GMPCPP microtubules
(Figure S2C). In principle, a tapered microtubule end structure
resulting from protofilaments of different lengths [27, 28] could
broaden the effective PSF width of the fitted end profile [29]. To
explore our sensitivity to any tapering, we simulated dynamic
microtubules with blunt and tapered ends [30] and analyzed
the resulting simulated movies (Figures S2D–S2F; Supple-
mental Experimental Procedures). We found that only taper
lengths larger than 180 nm lead to detectable broadening of
the effective PSF (Figures S2G–S2I). This means that, under
our conditions, the ends of growing microtubules have on
average end tapers shorter than ~180 nm. Furthermore, the
precision in determining the simulated microtubule end posi-
tion was estimated to be 4 nm along and 2 nm perpendicular
to the microtubule axis (Table S2; Supplemental Experimental
Procedures). The possible effects of tapering are discussed
further later.

The comet-like superaveraged EB1-GFP intensity profiles
could be well fitted by exponentially modified Gaussian func-
tions, i.e., a convolution of a monoexponential function with
a Gaussian PSF (Figure 2C). However, the start position of
the assumed monoexponentially decaying molecular density
distribution had to be a free-fitting parameter to produce
good fits and was found to increase with the growth velocity
up to ~100 nm behind the microtubule end (Figure 2D, black
points; Figure 2F). This shift, even if considerably smaller
than the apparent shift of the fluorescence intensity profile
peaks (Figures 2C and 2D, green points), was much larger
than our error and suggests that at the very end of growing
microtubules, a zone exists with a length of several tubulins
to which EB1 does not bind and that increases its size with
increasing growth velocity.

A similar growth velocity-dependent shift away from
the growing microtubule end was not observed for XMAP215-
GFP (Figures 2G–2L, see legend; Figure S2A). The fluores-
cence intensity profiles were well fitted by a simple function
(rep resenting a point source of fluorescence) combined with a
constant offset to one side (reflecting weaker binding to the
mature microtubule lattice), convolved with a Gaussian function (from now on called the Gauss-lattice model) (Figures 2 I and 2L). The XMAP215 position remained within a region of 10–20 nm behind the microtubule end, in good agreement with structured illumination microscopy measurements of chTOG at microtubule ends in fixed cells [25]. The small displacement from the microtubule end might reflect that XMAP215 binds in a defined orientation, which would position the C-terminally fused GFP at a certain distance from the microtubule end.

These results raise the question about the molecular mechanism causing EB1, in contrast to XMAP215, to be...
absent from the front of the growing microtubule. Two scenarios can be envisaged: either EB1 binds only slowly to freshly formed binding sites, causing a shift of the observed EB1 position due to delayed binding, or alternatively, the GTP hydrolysis cycle-linked EB1 binding sites could be absent in the very front region of the microtubule and would first have to be formed with measurable kinetics, causing the EB1 binding region itself to be displaced from the microtubule end.

Delayed EB1 Binding Cannot Explain Growth Velocity-Induced EB1 Profile Shifts
We first examined the contribution of EB1 binding kinetics to the position and shape of the observed EB1-GFP signal. Using single-molecule imaging at growing microtubule ends (10 µM EB1-GFP; Figure S3A), we measured the dissociation rate constant \( k_{\text{off}} = 3.4 \pm 0.2 \text{ s}^{-1} \) and estimated an association rate constant \( k_{\text{on}} \) of \( \sim 0.12 \text{ nM}^{-1}\text{s}^{-1} \) (Figure 3B, Supplemental Experimental Procedures). There is no indication of anomalously short events in either distribution, indicating that fluorescent blinking can be neglected. The bleaching rate was an order of magnitude slower (0.018 s\(^{-1}\)) (Figure S3B) and hence was also neglected. We determined the equilibrium dissociation constant \( K_D = 2 \times 10^{-6} \text{ M} \) by measuring a binding curve [16] for the concentration range of 0.125–800 nM EB1-GFP (Figure 3C) [16], showing noncooperative binding (Figure 3C inset); using this value and the \( k_{\text{off}} \) above gives a \( k_{\text{on}} \) of \( k_{\text{on}}/K_D = 0.15 \pm 0.01 \text{ nM}^{-1}\text{s}^{-1} \), consistent with the estimate above.

In the current kinetic model, EB1 binding sites (state \( B \) in Figure 3A) are generated as soon as tubulin is added to the growing microtubule end and then transform into the mature lattice (one conformational maturation step \( B \rightarrow C \) in Figure 3A) [3]. We extended this model by explicitly taking EB1-GFP binding and dissociation into account (using the measured rate constants \( k_{\text{on}} \) and \( k_{\text{off}} \), Figure 3A). A mathematical convolution of the solution of the relevant rate equations with the effective PSF (Supplemental Experimental Procedures) was then used to fit the EB1-GFP fluorescence intensity profile measured at the highest growth velocity of 95 nm/s (Figure 3D, blue curve). The poor fit demonstrates that slow binding of EB1-GFP cannot be solely responsible for the observed shift of the EB1-GFP fluorescence intensity profile.

Two-Step Model of EB Binding Site Formation and Decay
To test the second scenario of an additional kinetic step leading to a delayed formation of EB1 binding sites in the microtubule end region, we further extended our kinetic model (\( A \rightarrow B \) with \( k_1 \) in Figure 4A). The new microtubule conformation state \( A \) to which EB1 does not bind was assumed to form instantaneously with tubulin addition to the growing end. After calculating the modified rate equations and the resulting convolved spatial model, we performed a global fit to all EB1-GFP fluorescence intensity profiles: the kinetic rate constants \( k_1 \) and \( k_2 \) were shared for the three velocity conditions; \( k_{\text{on}}, k_{\text{off}}, \) and the growth velocities \( v_g \) were fixed to the experimentally determined values. Despite these constraints, a good global fit to all three EB1-GFP intensity profiles was possible (Figure 4B, black lines). Neglecting binding/unbinding kinetics did not allow a satisfactory global fit. This analysis provided the conformational maturation rate constants \( k_1 = 1.39 \pm 0.05 \text{ s}^{-1} \) and \( k_2 = 0.24 \pm 0.003 \text{ s}^{-1} \) (Table S1). Hence, the characteristic time for the formation of EB1 binding sites at a EB1-GFP concentration of 1 nM is \( \tau_1 = 1/k_1 = 0.7 \text{ s} \), and the lifetime of the EB1 binding sites is \( \tau_2 = 1/k_2 = 4 \text{ s} \), both of which are independent of the microtubule growth velocity.

The (nonconvolved) molecular EB1 distributions as calculated from the solution of the rate equations using the parameter values obtained from the fits (Figure 4C) show the highest density of EB1-GFP 80 nm (\( \sim 10 \) tubulin lengths) behind microtubule ends growing at 49 nm/s; this distance increases to 160 nm (\( \sim 20 \) tubulin lengths) at the higher growth velocity of 95 nm/s (Figure 4D, black points). These values (in contrast to those estimated in Figure 2F) result now from a mechanistic model explaining the shift of the EB1 binding sites away from the microtubule end. The kinetics of EB1-GFP binding have only a small, but nevertheless nonnegligible, contribution to the observed EB1-GFP position (Figure S4A). The molecular EB1-GFP distributions shown here extracted from fits to the fluorescence intensity profiles were similar to distributions obtained by a direct deconvolution of the intensity profiles with the experimentally measured PSF (Figure S4B), providing an independent validation of our kinetic model.

Finally, because four adjacent tubulins are required to form a high-affinity EB1 binding site, we investigated whether the nonbinding \( A \) states could be explained solely by tapering at the microtubule end (Figure S4C). Such tapered ends or “sheets” can be observed by electron microscopy [27] or in simulations [23]. Considering the maximum possible taper length in our experiments, we find that for “sheet”-like tapers, the resulting distribution of EB1 binding sites cannot reproduce the observed fluorescence profiles (Figure S4C, bottom).

In summary, this analysis establishes a kinetic two-step model of conformational maturation of growing microtubule ends, as monitored by EB1-GFP. The first maturation zone is characterized by lack of EB1 binding (see Discussion) and separates the bulk of EB1, which binds to the second maturation zone, from XMAP215, which binds to the very end of the microtubule. The two maturation rates (\( k_1 \) and \( k_2 \)) and hence the total “maturation time” (the sum of the lifetimes of both immature end states; see Supplemental Experimental Procedures) remain constant independent of microtubule growth velocity, leading to an increase of size of the maturation zones with increasing growth velocity (Figure 4E).

EB1 Decreases the Maturation Time of Growing Microtubule Ends
We next investigated the effect of EB1 on the two maturation transitions at microtubule ends. We performed experiments with increasing EB1-GFP concentrations and generated superaveraged normalized EB1-GFP fluorescence intensity profiles (Figure 5B). To exclude microtubule growth velocity effects, we chose microtubules with growth velocities of 95 ± 7 nm/s for all analyzed EB1-GFP concentrations. Increasing EB1-GFP concentrations shifted the fluorescence intensity peak toward the microtubule end and shortened the profiles (Figure 5B); this latter result was expected from the previous observation that the fission yeast EB1 homolog Mal3 decreased the lifetime of its binding site [16], indicating that this is a conserved property of EBs. Using our convolved two-step model with binding kinetics, we found that a global fit with shared rate constants \( k_1 \) and \( k_2 \) was not possible for these data. A good fit to the data was only possible when both rate constants were free-fitting parameters (Figure S5A),
Figure 2. Effect of Microtubule Growth Velocity on the EB1-GFP and XMAP215-GFP Fluorescence Intensity Profiles at Growing Microtubule Ends
(A) Superaveraged images of Cy5-microtubule ends growing at different mean growth velocities \( v_g \) in the presence of 1 nM EB1-GFP. Microtubules were selected for averaging according to their growth speed from experiments with 16.5–38 μM tubulin (see Figure S2 B).
(B and C) Superaveraged fluorescence intensity profiles of the Cy5-microtubule end (B) and EB1-GFP signal (C). Profiles contain information from 3,200 to 7,800 raw images in total.
(D) The start positions of the assumed monoexponential EB1-GFP density distributions (black points), relative to the microtubule end, determined from (F). Also shown are the apparent peak positions obtained from a cursory inspection of the profiles in (C).

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in which case both rates increased with increasing EB1-GFP concentration (Table S1).

We extended the model by considering an additional direct transition from the EB1-occupied binding site BE to the mature lattice state C (k3, Figure 5A); this allowed a global fit to the three normalized intensity profiles with shared constants k2 = 0.23 ± 0.01 s⁻¹ and k3 = 0.73 ± 0.01 s⁻¹ (Figure 5B; see Figure S5B for nonnormalized intensity profiles). This provides a kinetic explanation for the acceleration of the second microtubule maturation step, without necessitating varying rate constants: at higher EB1 concentrations, more binding sites are in the bound BE state with a faster decay rate. The observed acceleration of the first maturation step (k1) by EB1-GFP (Table S1) could be due to long-range effects that EB1 binding might have, such as zipping of protofilaments, which are not considered in our kinetic model. As a result of the altered formation and decay rates, the microtubule maturation time decreases with increasing EB1 concentration.

Figure 3. A One-Step Microtubule Maturation Model with Binding Kinetics Does Not Explain the EB1-GFP Intensity Profiles

(A) Simple one-step kinetic model of microtubule maturation and EB1 binding and a schematic of a microtubule growing from right to left. Different colors illustrate the two different states (B and C) of the microtubule; green dots indicate bound EB1 molecules. Binding and unbinding kinetics of EB1 are characterized by the rate constants kon and koff, respectively. The B state transforms at a rate k2 into the mature microtubule lattice conformation, C.

(B) Top: histogram of dwell times of single EB1-GFP molecules (10 pM) at growing Cy5-microtubule ends (20 µM tubulin). A mean dwell time of 290 ± 20 ms was obtained from a monoexponential fit. Bottom: histogram of waiting times between EB1-GFP binding events; a mean waiting time of 8.7 ± 0.6 s was obtained from a monoexponential fit.

(C) Average maximum EB1-GFP fluorescence intensities at growing Cy5-microtubule ends (20 µM tubulin) as a function of EB1-GFP concentrations. For each data point, intensities from at least 1,451 images were averaged. A fit to the data (red line) using a one-site binding model yields a dissociation constant KD of 22 ± 1 nM. Inset: enlarged view of the lowest concentrations, showing no indication of cooperative binding.

(D) The effect of EB1 binding kinetics on theoretical fluorescence intensity profiles. Green data points show the superaveraged EB1-GFP fluorescence intensity profile for an average growth speed of 95 nm/s. The solid lines show fits to the data using the one-step binding model without (red) and with (blue) inclusion of the EB1 binding kinetics (kon = koff/KD = 0.15 nM⁻¹s⁻¹ and koff = 3.4 s⁻¹; the effective point spread function was fixed at 175 nm in both cases).

Error bars are SE. See also Figure S3.

We extended the model by considering an additional direct transition from the EB1-occupied binding site BE to the mature lattice state C (k3, Figure S5A); this allowed a global fit to the three normalized intensity profiles with shared constants k2 = 0.23 ± 0.01 s⁻¹ and k3 = 0.73 ± 0.01 s⁻¹ (Figure S5B; see Figure S5B for nonnormalized intensity profiles). This provides a kinetic explanation for the acceleration of the second microtubule maturation step, without necessitating varying rate constants: at higher EB1 concentrations, more binding sites are in the bound BE state with a faster decay rate. The observed acceleration of the first maturation step (k1) by EB1-GFP (Table S1) could be due to long-range effects that EB1 binding might have, such as zipping of protofilaments, which are not considered in our kinetic model. As a result of the altered formation and decay rates, the microtubule maturation time decreases with increasing EB1 concentration.

(E and F) Calculated molecular density distributions derived from the fits to the Cy5-microtubule profiles (E) using an error function (black lines in B) and from the EB1-GFP profiles (F) using an exponentially modified Gauss model (black lines in C).

(G–L) As in (A)–(F), for Cy5-microtubule ends (11.3 µM tubulin) growing at different mean velocities in the presence of 150 nM XMAP215-GFP. The super-averaged profiles were generated from 1,600 to 3,400 raw images in total. As for the experiments with EB1-GFP, no significant changes in the microtubule end profiles were detected with increasing growth velocity (H and J). Fits (black lines) to the XMAP215-GFP data in (I) use a Gauss-lattice model. Error bars are SE. See also Figure S2.
Therefore, the calculated $B$ state molecular density distributions (Figure 5C, using the rate constants from the fit; Table S1) show a decrease of the peak position from 160 to 32 nm when the EB1 concentration increases from 1 to 50 nM (Figure 5D). Concomitantly, the number of tubulin dimers in states $A$ and $B$ (both bound and unbound with EB1) both decrease with increasing EB1-GFP concentration (Figure 5E). The values agree well with previous estimates for growing microtubules in cultured human cells [31].

Similar effects were not observed for XMAP215-GFP when its concentration was varied. A simple Gauss-lattice model could fit the fluorescence intensity profiles recorded over a range from 20 to 150 nM XMAP215-GFP (Figure 5C), and no significant changes of the molecular XMAP215 peak position (Figure 5D) were detectable.

EB1 and XMAP215 Do Not Influence Each Other’s Distinct Binding Sites

EB1 and XMAP215 have been reported to act synergistically on microtubule growth in vitro [19] but were reported to bind to independent sites in cells [25]; hence, we tested whether they affect each other’s localization to growing microtubule ends. We first generated superaveraged intensity profiles of 1 nM EB1-GFP alone and in the presence of 150 nM XMAP215 (Figures 6A and S2A). A global fit to the data with shared $k_1$ and $k_2$ rates indicates that the observed shift of the EB1 fluorescence profile away from the microtubule end can be explained solely as a direct consequence of an increase in microtubule growth velocity from 64 to 107 nm/s by XMAP215 (cf. Figure 4D, open circles). Therefore, in contrast to EB1, XMAP215 does not significantly affect maturation kinetics of the microtubule end ($k_1$ and $k_2$ were constant at 1.39 and 0.24 s$^{-1}$, respectively). When comparing the peak position of 20 nM XMAP215-GFP alone and in the presence of 150 nM EB1, only a minimal shift of the XMAP215 position within our resolution limit could be detected (Figures 6B and S2A).

These results agree with our findings that EB1 and XMAP215 bind to different locations at growing microtubule ends. The different nature of the binding sites of these two proteins is further demonstrated by the observation that XMAP215-GFP binds only to the very ends of microtubules growing in the presence of GTP$_\gamma$S, whereas EB1-GFP is known to decorate these microtubule along their entire length with high affinity (Figure 6C) [16]. Therefore, XMAP215 binds to its binding site in a tubulin-nucleotide state-independent manner, in clear contrast to EB1.
contrast to EB1. In conclusion, low growth velocities and high EB1 concentrations shorten the molecular distribution profiles of EB1 and shift its peak closer to the microtubule end, where XMAP215 is located at a distinct, focused binding region (Figure 6D).

Promotion of Microtubule Maturation by EB1 Accelerates the Kinetics of Catastrophe Induction

The density of binding sites for the fission yeast EB1 homolog Mal3 and human EB3 has been shown to decrease before catastrophe [15, 21], suggesting that these sites might have a stabilizing role for the growing microtubule. In contrast, XMAP215 binding sites are not lost before catastrophe but exist throughout the transition from polymerization to depolymerization (Figure S6) [20]. Therefore, EB1 proteins are unique reporters for structural changes leading to catastrophe.

We decided to examine how microtubule maturation times relate to the kinetics of the transition from polymerization to depolymerization to gain insight into the mechanism of catastrophe induction.

We tracked dynamic microtubules in the presence of EB1-GFP and generated plots of the averaged microtubule end position and the averaged total EB1 intensity in end regions as a function of time around catastrophe events for different microtubule growth velocities and EB1-GFP concentrations (from more than 44 catastrophes per condition; see Supplemental Experimental Procedures). The time course of the averaged microtubule end position revealed that the transition from growth to shrinkage was not instantaneous: it occurred over a period of several seconds, resulting in the appearance of a transient pause (Figures 7A and 7B). We found that this pause time was apparently independent of the microtubule growth velocity (Figure 7A) but decreased with increasing EB1 concentration (Figure 7B). In striking similarity, the rate of the loss of EB1 binding sites before catastrophe [15] was also independent of the microtubule growth velocity (Figure 7C) and increased with increasing EB1 concentration (Figure 7D).

This reveals an interesting and so far unreported relation between the kinetics of microtubule end maturation, which are influenced by the EB1 concentration but not by the growth velocity, and the kinetics from growth to shrinkage during catastrophe. Strikingly, for each condition, the observed pause times were very similar to the total maturation times.
Similarly, the EB1 binding site decay times before catastrophe were also proportional to the maturation times (Figure 7E, bottom). Microtubules growing at different growth velocities (Figures 7A and 7C) have different lengths of end regions (Figure 4E); hence, the pause times, as with the EB1 binding site decay times, do not depend on the length of these end regions. Finally, we observed that fast microtubule shrinkage began typically when the fraction of observed EB1 binding sites was around 20% of its steady-state level (Figures 7C and 7D). In contrast to EB1, the presence of saturating amounts of XMAP215-GFP had no effect on the pause time compared to microtubules grown in the presence of a low amount of EB1-GFP (Figure S6).

Taken together, these results suggest that microtubule maturation kinetics (but not the lengths of the capping zones) determine the kinetics of catastrophe induction. Catastrophes appear to be induced when the duration of an episode of slow growth (or a pause in growth) is long enough to lead to a loss of the majority of the EB1 binding sites in the microtubule end (see Discussion). Therefore, EB1 increases the catastrophe frequency [4, 17–21] (Figure S7A), most likely by accelerating conformational transitions in the growing microtubule end that shorten the lifetime of a protective end structure (Figures 7F and 7G).

Discussion

Using convolved model fitting to averaged fluorescence intensity profiles [32], we have determined the molecular density distributions of EB1 along microtubule end regions in the nanometer range. The distributions reveal that two consecutive conformational maturation steps take place in...
Figure 7. The Effect of Microtubule Growth Velocity and EB1 Concentration on Pause Times and EB1 Intensity Decay Rates before Catastrophes

(A) Averaged microtubule end position aligned at the catastrophe time point (see Supplemental Experimental Procedures) at different tubulin concentrations and hence growth velocities (50 nM EB1-GFP). The gray lines indicate growth pauses before catastrophe (Supplemental Experimental Procedures).

(C) Normalized averaged EB1-GFP intensity-time profiles corresponding to (A). Solid lines are exponential fits to the data. The dashed and dotted horizontal lines give the average residual EB1-GFP intensity at catastrophe and error, respectively.

(B and D) As in (A) and (C), for varying EB1-GFP concentrations (20 μM tubulin). Note that the pause time at 50 nM EB1-GFP in (B) is comparable to the pause times shown in (A) at the same EB1-GFP concentration and different tubulin concentrations.

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the microtubule end region, formally similar to an earlier model with consecutive nucleotide hydrolysis cycle-dependent steps taking place in the growing microtubule end [53]. Here, high-affinity binding sites for EB1 are formed by the first maturation step and are then destroyed by the second. This explains why the peak of the EB1 density distribution is displaced from the microtubule end position. The intrinsic formation time of the EB1 binding site at the microtubule end (at low EB1 concentration) was approximately five times shorter than its lifetime \( \tau_2 \) of 4 s; this latter value is in the range of previous estimates [4, 5].

EB1 binding accelerates both maturation steps, shortening its own binding region and shifting it closer to the microtubule end. Maturation kinetics are not altered by the microtubule growth velocity; hence, fast-growing microtubules show more stretched EB1 density distributions, and the effect of EB1 on maturation is more obvious. In contrast to EB1, XMAP215 is always close to the microtubule end, in agreement with previous work [25].

What could be the structural reason for EB1 not binding to the very front region? A subnanometer-resolution structure of the calponin homology domain of Mal3 in complex with GTP+S microtubules revealed that Mal3 contacts four adjacent tubulin dimers, one of the contacts involving the \( \beta \)-tubulin H3 helix, which is close to the GTP hydrolysis site [15]. Interactions with all contact areas were shown to be important for Mal3 binding. As a consequence, the absence of lateral contacts between protofilaments, for example due to microtubule end tapering [protofilaments with different lengths] [18, 28, 29], is expected to decrease the density of EB1 binding sites at the very end of microtubules. Interestingly, under our conditions, the end taper length extracted from the Cy5-microtubule end intensity profiles was on average less than 180 nm (our resolution limit for the detection of tapered ends; Figures S2D–S2I); this is less than other previous reports based on fluorescence microscopy imaging [29, 30] but roughly in agreement with previous electron microscopy data [18, 28] (note that longer taper lengths have been reported under some electron microscopy conditions [27]). However, geometrical arguments show that tapering is unlikely to be the only reason for the absence of EB1 binding sites close to the microtubule end (A sites in our kinetic model) (Figure S4C). Alternatively, or additionally, an unfavorable binding site conformation, due to either protofilament bending or GTP hydrolysis-linked conformational changes of the \( \beta \)-tubulin H3 helix, might prevent EB1 binding to the very front of the growing microtubule. In the future, it will be interesting to apply higher-resolution methods to try to characterize in more detail the structural nature of the A sites detected by our analysis of averaged intensity profiles.

Because EB1 binds with high affinity to microtubules grown in the presence of the GTP analog GTP-\( \gamma \)S, but much less so to microtubules grown in the presence of another GTP analog, GMP-CPP [16] (Figure 6C), it is not straightforward to conclude that the high-affinity binding site for EB1 in the growing microtubule end region corresponds to the GTP and/or GDP+Pi state. However, it is certain that EB1 binds with high affinity to a transiently existing conformational state that exists as part of the GTP hydrolysis cycle before the mature GDP lattice is formed; this might indeed be the GTP and/or GDP+Pi state. Because EB1 binds close to the GTP hydrolysis site in the microtubule, it is likely that it accelerates GDP lattice formation by accelerating GTP hydrolysis and/or associated conformational changes in lattice-incorporated tubulin, promoting microtubule maturation.

We found that before a catastrophe, microtubule growth slows down for several seconds before depolymerization starts, giving rise to the appearance of pauses of growth. Interestingly, the pause time depended on EB1 concentration, but not on the microtubule growth velocity, and was very similar to the microtubule maturation time (Figure 7E). This reveals a novel link between the kinetics of the formation of a mature tube during microtubule growth and the kinetics of the transition from growth to shrinkage. Depolymerization started once the fraction of tubulins forming EB1 binding sites (and possibly also the tubulins forming the first maturation zone) fell below a threshold value, suggesting the requirement of a minimal density of stabilizing cap sites for continued growth. The observed pauses might reflect a stochastic slowdown of growth as a result of growth fluctuations [34]. Furthermore, we observed that the catastrophe time (inverse of the catastrophe frequency; Figure S7A) was also proportional to the microtubule maturation time (Figure 7F). Therefore, the acceleration of microtubule end maturation by EB1 can also explain the catastrophe-promoting effect of EB1 (Figure S7A) [4, 17–21].

Recently, EB1 and XMAP215 were reported to promote microtubule growth synergistically [19]. Based on the known structures of the EB1 binding site on microtubules [15] and of soluble tubulin in complex with the microtubule binding domain of a XMAP215 homolog [24], it has been suggested that EB1 might promote the activity of XMAP215 by straightening protofilaments [19]. Such straightening is expected to release XMAP215 from freshly incorporated tubulin subunits [24]. Our observation that EB1 shifts its own binding region several tens of nanometers toward the microtubule end could indicate that these two proteins act synergistically when being in closer proximity. This might explain why the Drosophila homologs of EB1 and XMAP215 were observed to require sentinel as a bridging protein for synergy [20], possibly because it brings the Drosophila homologs of EB1 and XMAP215 closer together.

How do our in vitro results compare to the situation in the living cell? Recently, a shift of \( \sim 100 \) nm between the peaks of the fluorescence intensity profiles of EB1 and chTOG at growing microtubule ends was observed in HeLa cells by structured illumination microscopy [25]. Due to the asymmetry of the EB1 profile, this is most likely an overestimate for the distance between the molecular profiles, as can be shown by convolved model fitting to the reported data (Figure S7B), even if the PSF in structured illumination experiments is narrower than in standard TIRF microscopy.

In conclusion, convolved model fitting to averaged TIRF microscopy data allowed us to extract molecular density distributions of EB1 at growing microtubule end regions for the first time at the nanoscale. These distributions reveal
that microtubule ends mature in at least two steps and that EB1 catalyzes both steps. The EB1-induced decrease in maturation time correlates with a reduction in pause time between the transition from growing to shrinking microtubules just before catastrophe. In addition to the recent progress in our understanding of the molecular mechanism of the role of EB1 as the major recruitment factor for plus-end-tracking proteins [2, 4, 13, 35], the results presented here advance our understanding of the mechanism underlying EB1’s second function as a catalyst of nanoscale structural rearrangements at the growing microtubule end that play an important role for regulating microtubule dynamics.

Experimental Procedures

Protein expressions and purifications were performed using standard methods. For details, see Supplemental Experimental Procedures.

The in vitro reconstitution of microtubule end tracking was performed as described previously [36]. For simultaneous dual-color time-lapse imaging of the Cy5 and GFP channel, images with 300 ms exposure time were simultaneously recorded every 0.5 s using a TIRF microscope illuminating with 488 and 640 nm lasers. A complete description of the experimental setup is given in the Supplemental Experimental Procedures. Single-molecule imaging of EB1-GFP and binding curve measurements were performed as described previously [16]. Microtubule ends were tracked automatically using a modified and extended version of a previously published program [28]. Averaged fluorescence profiles were analyzed using convolved model fitting [32]. A complete description is given in the Supplemental Experimental Procedures for the microtubule tracking, alignment, and averaging, as well as the various mathematical models used.

Supplemental Information

Supplemental Information includes seven figures, two tables, Supplemental Experimental Procedures, and two movies and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.12.042.

Author Contributions

S.P.M., N.I.C., G.B., and T.S. developed the study, analyzed data, and wrote the paper. S.P.M. designed and performed most experiments and coordinated interactions within the interdisciplinary team. G.B. wrote the MATLAB program for microtubule end tracking and image averaging and other programs. N.I.C. was responsible for microscopy development, implementing kinetic models, and writing programs. N.G. wrote the program to simulate microtubule tracking, alignment, and averaging, as well as the various mathematical models used.

Supplemental Information

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