EB1 promotes microtubule dynamics by recruiting Sentin in *Drosophila* cells

Wenjing Li,[1] Tomohiro Miki,[1] Takashi Watanabe,[2] Mai Kakeno,[2] Ikuko Sugiyama,[2] Kozo Kaibuchi,[2] and Gohta Goshima[1]

[1]Division of Biological Science, Graduate School of Science, and [2]Department of Cell Pharmacology, Graduate School of Medicine, Nagoya University, Nagoya 464-8601, Japan

Highly conserved EB1 family proteins bind to the growing ends of microtubules, recruit multiple cargo proteins, and are critical for making dynamic microtubules in vivo. However, it is unclear how these master regulators of microtubule plus ends promote microtubule dynamics. In this paper, we identify a novel EB1 cargo protein, Sentin. Sentin depletion in *Drosophila melanogaster* S2 cells, similar to EB1 depletion, resulted in an increase in microtubule pausing and led to the formation of shorter spindles, without displacing EB1 from growing microtubules. We demonstrate that Sentin's association with EB1 was critical for its plus end localization and function. Furthermore, the EB1 phenotype was rescued by expressing an EBN-Sentin fusion protein in which the C-terminal cargo-binding region of EB1 is replaced with Sentin. Knockdown of Sentin attenuated plus end accumulation of Msps (mini spindles), the orthologue of XMAP215 microtubule polymerase. These results indicate that EB1 promotes dynamic microtubule behavior by recruiting the cargo protein Sentin and possibly also a microtubule polymerase to the microtubule tip.

Introduction

Microtubules are dynamic polymers made from α/β-tubulin dimers and are crucial for various cellular events, such as cell division, polarization, motility, or organelle transport (Desai and Mitchison, 1997). Conventionally, microtubule dynamics cycles are divided into four events: growth, shrinkage, catastrophe (polymerization to depolymerization transition), and rescue (depolymerization to polymerization transition; Mitchison and Kirschner, 1984; Horio and Hotani, 1986; Kinoshita et al., 2001). In addition, a "pause" constitutes another state in vivo, where neither rapid polymerization nor depolymerization is observed for certain periods of time (Dhamodharan and Wadsworth, 1995; Desai and Mitchison, 1997; Rogers et al., 2002; Sousa et al., 2007; Yao et al., 2008). In cells, dynamic microtubules are generated with the contribution of nontubulin proteins, particularly those working at the plus ends of microtubules (Howard and Hyman, 2007; Akhmanova and Steinmetz, 2008). The Dis1/XMAP215 family proteins, including yeast Dis1/Stu2, fly Msps (mini spindles), frog XMAP215, and mammalian ch-TOG (colonic and hepatic tumor overexpressed), have tubulin-binding TOG domains, and XMAP215 was shown to promote microtubule growth by processively adding tubulin dimers onto the plus ends and also catalyze the reverse reaction, namely the removal of tubulin from the end, which leads to the promotion of microtubule shrinkage (Kerssemakers et al., 2006; Howard and Hyman, 2007; Brouhard et al., 2008; Slep, 2010). Kinesin-13 is a microtubule-depolymerizing kinesin (Desai et al., 1999; Moore and Wordeman, 2004; Rogers et al., 2004a), and its inhibition leads to the suppression of catastrophe and formation of longer microtubules in spindles (Goshima and Vale, 2003; Goshima et al., 2005b; Mennella et al., 2005; Ohi et al., 2007). Interestingly, the essential features of physiological microtubule dynamics were reconstituted by mixing just tubulin, XMAP215, and Kinesin-13 (Kinoshita et al., 2001). Cytoplasmic linker protein (CLIP)–associated proteins (CLASPs; Mast or Orbit in fly) are another class of proteins containing the TOG-like domain and were recently shown to increase rescue and decrease catastrophe frequency (Al-Bassam et al., 2010). CLIPs (CLIP-190 in fly) promote microtubule growth in some cell types (Brunner and Nurse, 2000; Komarova et al., 2002) but may not in others (Dzhindzhev et al., 2005; Goshima et al., 2007).

Correspondence to Gohta Goshima: goshima@bio.nagoya-u.ac.jp

Abbreviations used in this paper: CLASP, CLIP-associated protein; CLIP, cytoplasmic linker protein; fps, frame per second; mRFP, monomeric RFP; UTR, untranslated region.

The Rockefeller University Press $30.00
J. Cell Biol. Vol. 193 No. 6 973–983
www.jcb.org/cgi/doi/10.1083/jcb.201101108

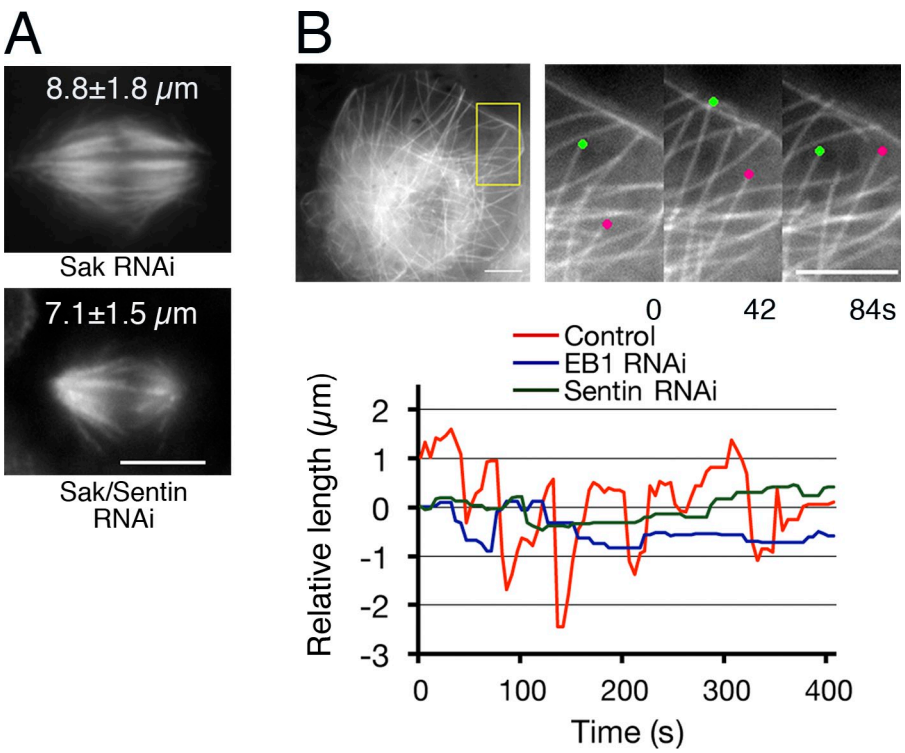

Figure 1. **Sentin, a novel regulator of microtubule dynamics.** (A) A shorter metaphase spindle was formed after the simultaneous RNAi knockdown of Sentin and Sak, a factor essential for centrosome formation (± SD; n = 12 and 24). (B) Microtubule plus end dynamics was suppressed by Sentin or EB1 RNAi. (top) A control cell that has many dynamic cytoplasmic microtubules during the interphase (the area encircled with yellow is magnified and the plus ends of two microtubules are marked with pink and green dots). (bottom) Life history plot of a representative microtubule after each RNAi treatment, in which microtubule length from the arbitrary chosen point is plotted over time. Also see Video 1 and Table I. Bars, 5 μm.

The EB1 family is referred to as the master regulator or lynchpin of microtubule plus end proteins because it directly and autonomously binds to the growing tips of microtubules via its N-terminal region and recruits various cargo proteins, including CLIPs, CLASPs, and Kinesin-13, to the tip via its C-terminal region (Akhmanova and Steinmetz, 2008; Slep, 2010). Although the key features of microtubule dynamics can be reconstituted with only XMAP215 and Kinesin-13 (Kinoshita et al., 2001), EB1 depletion in cells or frog extracts severely dampens microtubule plus end dynamics despite the presence of XMAP215 and Kinesin-13 (Rogers et al., 2002; Tirnauer et al., 2002). However, its exact molecular action toward microtubule plus end dynamics is largely unclear. For example, several reconstitution assays using purified recombinant EB1 led to inconsistent conclusions regarding its effect on microtubule catastrophe and growth (Bieling et al., 2007; Manna et al., 2008; Vitre et al., 2008; Dixit et al., 2009; Komarova et al., 2009; Zhu et al., 2009).

The *Drosophila melanogaster* S2 cell line is an excellent model system to study microtubule plus end dynamics because RNAi is very efficient and high-resolution microscopy can be applied to this cell line (Rogers et al., 2002; Goshima and Vale, 2003). Phenotypic classification of the factors responsible for microtubule dynamics has been extensively performed in RNAi-based studies (Rogers et al., 2002; Brittle and Ohkura, 2005; Goshima et al., 2005b, 2007; Sousa et al., 2007), which might not be easily performed in mammalian cells that often use multiple paralogous genes redundantly. For example, the microtubule phenotype has been investigated after coknockdown of two, but not all three, EB1 family members in mammals (Komarova et al., 2009). In S2 cells, EB1 or XMAP215^{Msps} RNAi knockdown increases the pause during interphase and results in the formation of shorter spindles (Rogers et al., 2002; Brittle and Ohkura, 2005). Kinesin-13^{Klp10A} RNAi decreases catastrophe frequency in interphase (Mennella et al., 2005), leads to formation of extremely long asters, and increases spindle length (Goshima and Vale, 2003; Rogers et al., 2004a). In this paper, we show that the novel protein Sentin is the dominant cargo of EB1 for promoting microtubule plus end dynamics and is also responsible for XMAP215^{Msps} accumulation at the growing microtubule ends and, thus, provide a new insight into the mechanism of EB1-dependent regulation of microtubule plus ends.

Results and discussion

Sentin is required for suppressing microtubule pause in S2 cells

In a genome-wide RNAi screen using S2 cells, we identified several genes whose knockdown leads to the shortening of the metaphase spindle, which was similar to EB1 or XMAP215^{Msps} RNAi (Goshima et al., 2007). Among them, we were interested in one uncharacterized gene, *CG9028/ssp2*, which we named "*Sentin*" from the Japanese word Sentan (tip) in the present study. After RNAi treatment, Sentin levels were reduced by 90% (Fig. S1 A), and the metaphase spindle length was 72% (n = 39) of the control. Centrosome/centriole-free spindles constructed using Sak (Plk4) RNAi (Bettencourt-Dias et al., 2005) were also shorter in the absence of Sentin than in the control cells (Fig. 1 A). Thus, the formation of shorter spindles is likely to be caused by the presence of shorter microtubules in the Sentin-depleted spindles (Goshima and Scholey, 2010).

We investigated microtubule polymerization dynamics after Sentin RNAi in S2 cells expressing GFP-tubulin. Similar to the EB1- or XMAP215^{Msps}-depleted cells (Rogers et al., 2002;

Table I. **Kinetic parameters of microtubule polymerization dynamics after Sentin RNAi in S2 cells**

Parameters	RNAi		
	Control	EB1	Sentin
Growth rate (μm/min)	5.3 ± 1.4	4.5 ± 0.8	3.5 ± 0.5
	5.3 ± 3.1^{m}	4.4 ± 2.5^{m}	3.5 ± 2.0^{m}
Shrink rate (μm/min)	11.3 ± 1.9	7.9 ± 1.4	7.7 ± 2.0
	11.1 ± 6.6^{m}	7.5 ± 4.9^{m}	7.4 ± 6.0^{m}
Catastrophe frequency (s^{-1})	0.011 ± 0.003	0.005 ± 0.001	0.005 ± 0.001
Rescue frequency (s^{-1})	0.005 ± 0.002	0.002 ± 0.001	0.003 ± 0.001
Time in growth (%)	39.0 ± 11.5	14.1 ± 8.2	20.8 ± 11.1
Time in shrink (%)	19.7 ± 5.0	7.9 ± 3.1	8.0 ± 1.6
Time in pause (%)	41.3 ± 10.5	78.0 ± 10.8	71.2 ± 11.7

Means ± SD. SD represents either microtubule to microtubule (marked with an m) or cell to cell (others) variability. *n* = 84 microtubules and 5 cells for control, 83 microtubules and 5 cells for EB1, and 80 microtubules and 5 cells for Sentin.

Brittle and Ohkura, 2005), the interphase microtubules, including those away from the cell edge, were less dynamic in the absence of Sentin and in a pause state for the majority of the time (Figs. 1 B and S1 B, Video 1, and Table I). Astral microtubules during metaphase were also less dynamic after Sentin RNAi and were frequently in the pause state (Table II and Video 2). The presence of astral microtubules is different from the case of EB1 RNAi, in which asters are nearly absent (Rogers et al., 2002); this is probably because EB1 has additional roles, for example, in centrosomal microtubule nucleation or the protection of growing microtubules from depolymerases (Vitre et al., 2008; Komarova et al., 2009). We conclude that Sentin, like EB1 and XMAP215^{Msps}, is necessary to promote microtubule dynamics in S2 cells.

Sentin is a microtubule plus end–tracking protein

Sentin is a 982 aa protein that possesses no recognizable domains from which the molecular activity of this protein could be deduced. Our BLAST (Basic Local Alignment Search Tool) searches identified clear homologues in insects but not in vertebrates. However, *Drosophila* spindle/microtubule-related proteins are generally conserved but their amino acid sequences are often highly diverged from those in vertebrates, and the identification of orthologues solely by computer-based search protocols is sometimes challenging (e.g., Goshima et al., 2007; Przewloka et al., 2007; Uehara et al., 2009; Stevens et al., 2010). It is therefore possible that there is a functional Sentin homologue in mammals that has not been identified or has been already identified without recognition.

To understand the function of Sentin, we first determined its localization in S2 cells. We generated a cell line possessing the inducible GFP-Sentin fusion gene and confirmed that GFP-Sentin rescues the short spindle phenotype that appeared after endogenous Sentin was depleted using RNAi targeting the 3′-untranslated region (UTR; Fig. S2 A). Time-lapse microscopy and costaining with microtubules or EB1 indicated that GFP-Sentin tracks microtubule plus ends during interphase and metaphase (Fig. 2 A and Video 3). This localization was also confirmed by immunostaining endogenous Sentin with polyclonal antibodies (Fig. 2 B). Fainter GFP-Sentin signals were also detected along microtubules, particularly when the expression level was high, suggesting that GFP-Sentin has weak affinity to microtubules. Some EB1-binding proteins, such as CLASPs (Lemos et al., 2000), are localized at the kinetochore in the absence of microtubules in mitosis. However, kinetochore localization was not detected for Sentin when microtubules were depolymerized by colcemid (Fig. S2 B). We concluded that Sentin is a novel microtubule plus end–tracking protein that behaves similarly to EB1 during the cell cycle.

Table II. **Kinetic parameters of centrosomal microtubule polymerization dynamics in mitosis**

Parameters	RNAi		
	Control	Sentin	Kinesin-13^{Klp10A}
Growth rate (μm/min)	10.8 ± 1.6	8.8 ± 2.4	9.8 ± 1.3
	10.8 ± 2.1^{m}	8.7 ± 4.0^{m}	9.7 ± 2.5^{m}
Shrink rate (μm/min)	19.6 ± 4.4	21.6 ± 4.9	40.2 ± 8.7
	19.5 ± 5.2^{m}	21.7 ± 6.5^{m}	40.2 ± 12.0^{m}
Catastrophe frequency (s^{-1})	0.035 ± 0.006	0.021 ± 0.008	0.025 ± 0.007
Time in growth (%)	53.1 ± 8.6	34.7 ± 15.3	62.7 ± 8.4
Time in shrink (%)	32.0 ± 7.5	17.8 ± 7.3	18.9 ± 6.0
Time in pause (%)	14.9 ± 11.1	47.5 ± 17.7	18.4 ± 10.6

Means ± SD. SD represents either microtubule to microtubule (marked with an m) or cell to cell (others) variability (20 microtubules and 10 cells).

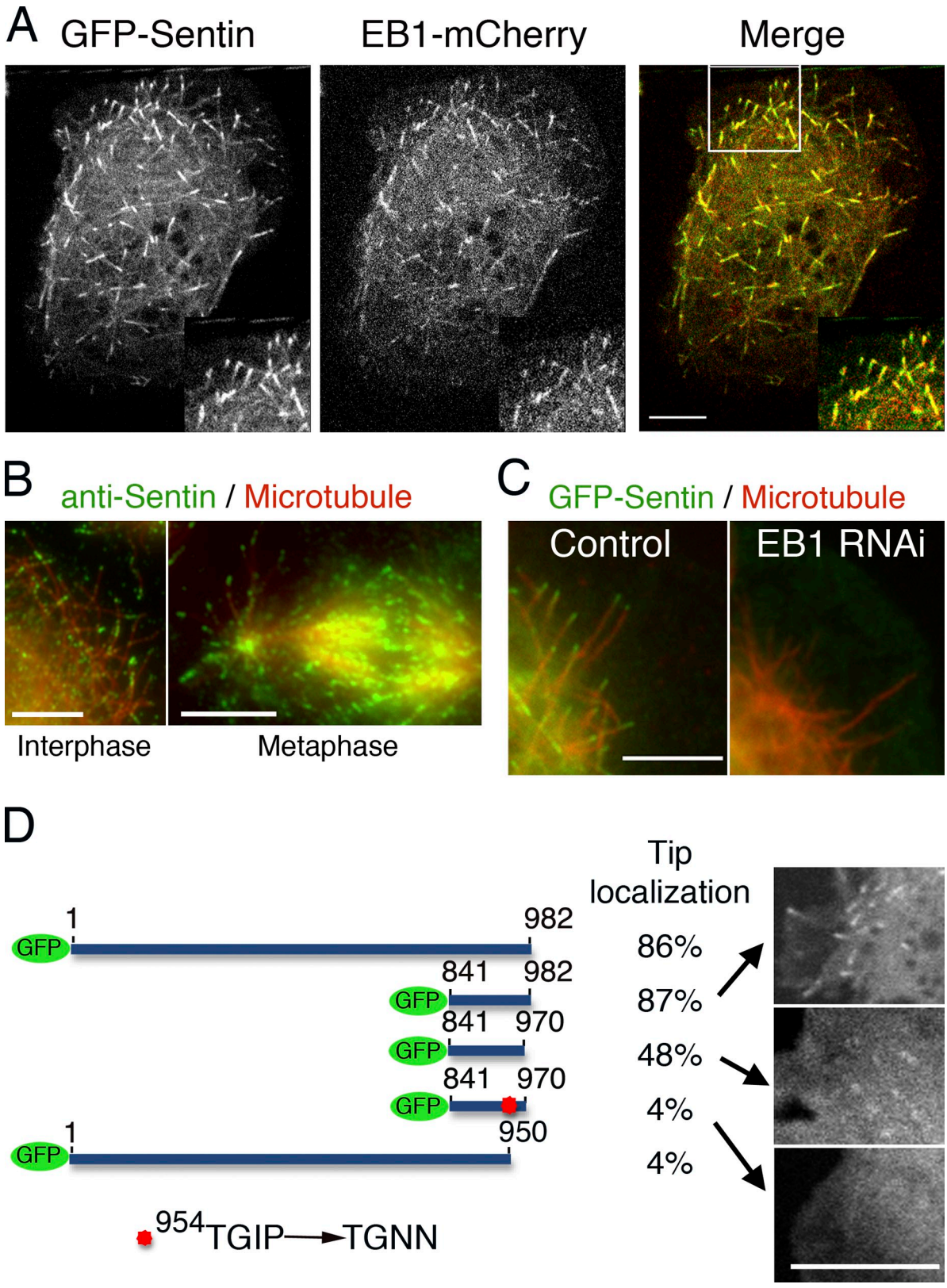

Figure 2. **Sentin is a microtubule plus end–tracking protein.** (A) GFP-Sentin (green) colocalized with EB1-mCherry (red) at the growing microtubule plus ends. The area boxed in white is magnified and placed at the bottom right. Also see Video 3. (B) The microtubule plus end localization of Sentin was revealed using a polyclonal anti-Sentin antibody. (C) Cells expressing GFP-Sentin were fixed and stained with microtubules. Microtubule tip localization was detected for 75% of the microtubules in the control cells ($n = 5$), whereas only 28% were observed after EB1 RNAi ($n = 9$). (D) A series of Sentin truncations revealed that the C-terminal region is essential and sufficient for plus end tracking. Frequency (percentage) of tip localization on growing microtubules for each Sentin fragment is presented (>50 microtubules analyzed). GFP images are shown on the right. Bars: (A and D) 10 µm; (B and C) 5 µm.

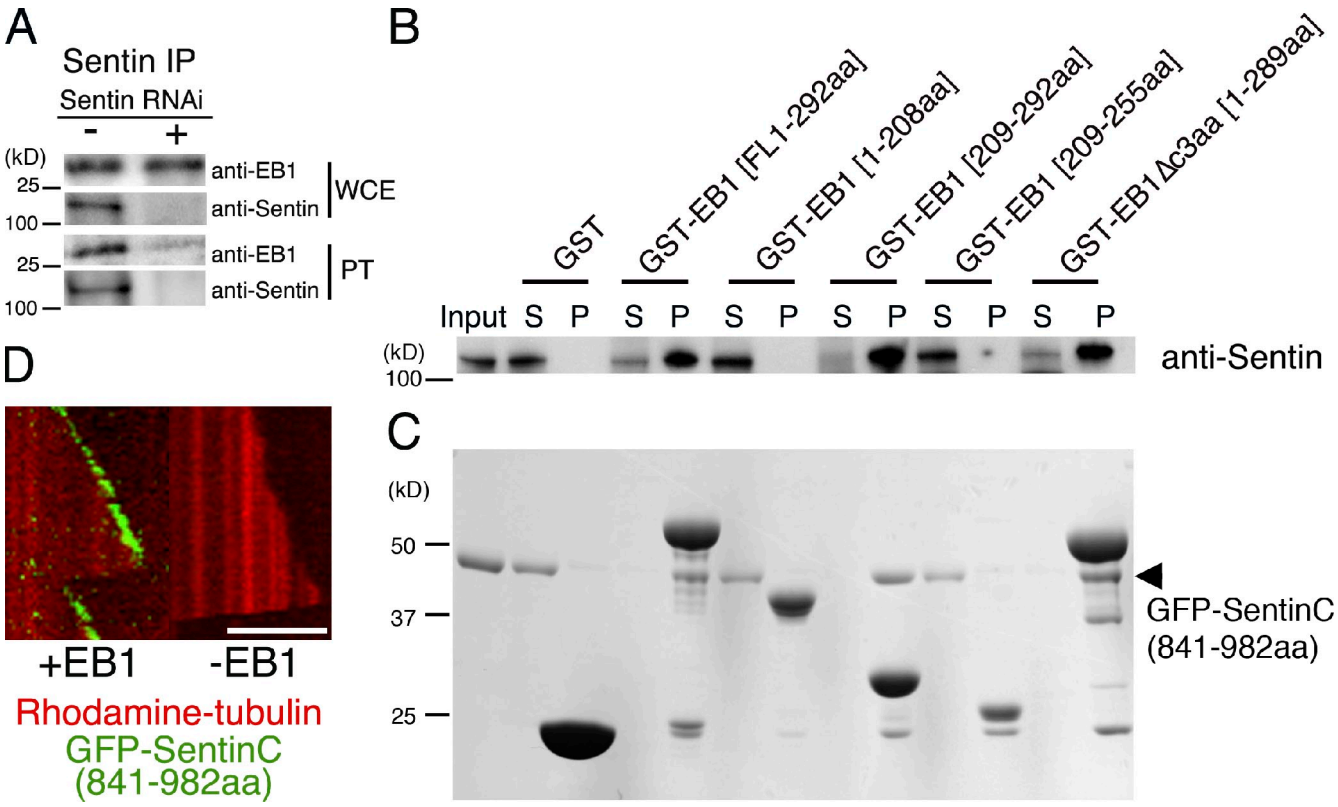

Figure 3. **Sentin interacts with EB1.** (A) Coimmunoprecipitation of endogenous EB1 and Sentin. Sentin was immunoprecipitated with the anti-Sentin antibody followed by detection of EB1 and Sentin by each polyclonal antibody (PT, pellet). Extracts of control S2 cells (−) and those depleted of Sentin by RNAi (+) were used (WCE, whole cell extracts). (B and C) Sentin interacts with EB1. GST pull-down assay using several truncations of GST-EB1 and S2 cell extracts (B) or purified His-GFP-SentinC (841–982 aa). Sentin binds to the C-terminal region of EB1, as revealed by immunoblotting (B) or Coomassie blue staining (C). S, supernatant after the beads were incubated with S2 extracts or recombinant Sentin. P, beads after washing (2.5-fold or equal amount loaded compared with the supernatant in B or C, respectively). (D) Kymographs showing EB1-dependent tracking of His-GFP-SentinC (841–982 aa) at the growing plus ends of microtubules (red) in the in vitro plus end–tracking assay. Bar, 5 µm. Also see Video 4.

Sentin is an EB1 cargo protein

We tested whether Sentin is localized to the plus ends by binding to EB1. First, we found that the plus end accumulation of GFP-Sentin is attenuated after EB1 RNAi (Fig. 2 C). Second, we determined the domain responsible for the plus end tracking of Sentin by making several truncated constructs (Fig. 2 D). The plus end localization was observed for the last 142-aa fragment (841–982 aa). Further 12-aa deletion from the C terminus (841–970 aa) partially abolished the localization, suggesting that both 841–970 and 971–982 regions are important for tracking. Sentin contains SxIP-like motifs that were recently shown to bind directly to the C terminus of EB1 (Honnappa et al., 2009). We mutated a TGIP sequence at the 954 aa residue to TGNN and expressed the 841–970 (TGNN) construct and found that it rarely localizes to the tip (Fig. 2 D). Third, when Sentin was immunoprecipitated from S2 cell extracts using the polyclonal antibody, EB1 was coprecipitated (Fig. 3 A). Fourth, when a pull-down assay was performed using purified GST-EB1 and S2 extracts, Sentin was found to be associated with GST-EB1 (Fig. 3 B). A similar level of association was also observed for an EB1ΔC construct that lacks the last 3 aa and cannot bind to the CAP (cytoskeleton-associated protein) Gly domain (Komarova et al., 2005), but this association was lost after the deletion of the C-terminal 37 aa that are known to affect the binding of other SxIP motif-containing proteins (Slep et al., 2005; Honnappa et al., 2009). Fifth, we purified the C-terminal 142 aa of Sentin (841–982) tagged with GFP and showed its direct binding to GST-EB1C (209–292 aa) but not to GST-EB1N (1–208 aa) in vitro (Fig. 3 C). Finally, we used the in vitro microtubule plus end–tracking assay (Bieling et al., 2007) and found that the C-terminal fragment (841–982 aa) tagged with GFP tracked the growing plus ends of microtubules in an EB1-dependent manner (Fig. 3 D and Video 4). Altogether, we concluded that Sentin is a new EB1 cargo protein.

Sentin recruitment is needed for EB1 to promote microtubule dynamics

Several EB1 cargo proteins have been identified, but for virtually all of these proteins, it is unknown whether binding to EB1 is essential for their intracellular functions (Rogers et al., 2004b; Goshima et al., 2005a; Mennella et al., 2005; Moore et al., 2005; Akhmanova and Steinmetz, 2008). To directly test whether EB1 binding is required for the function of Sentin, we performed a rescue experiment in S2 cells using truncated genes. The constructs that failed to accumulate at the plus ends (1–950 or 1–940 aa) could not rescue the short spindle or the pause-rich interphase phenotype of Sentin RNAi, and importantly, plus end localization and rescue were observed when

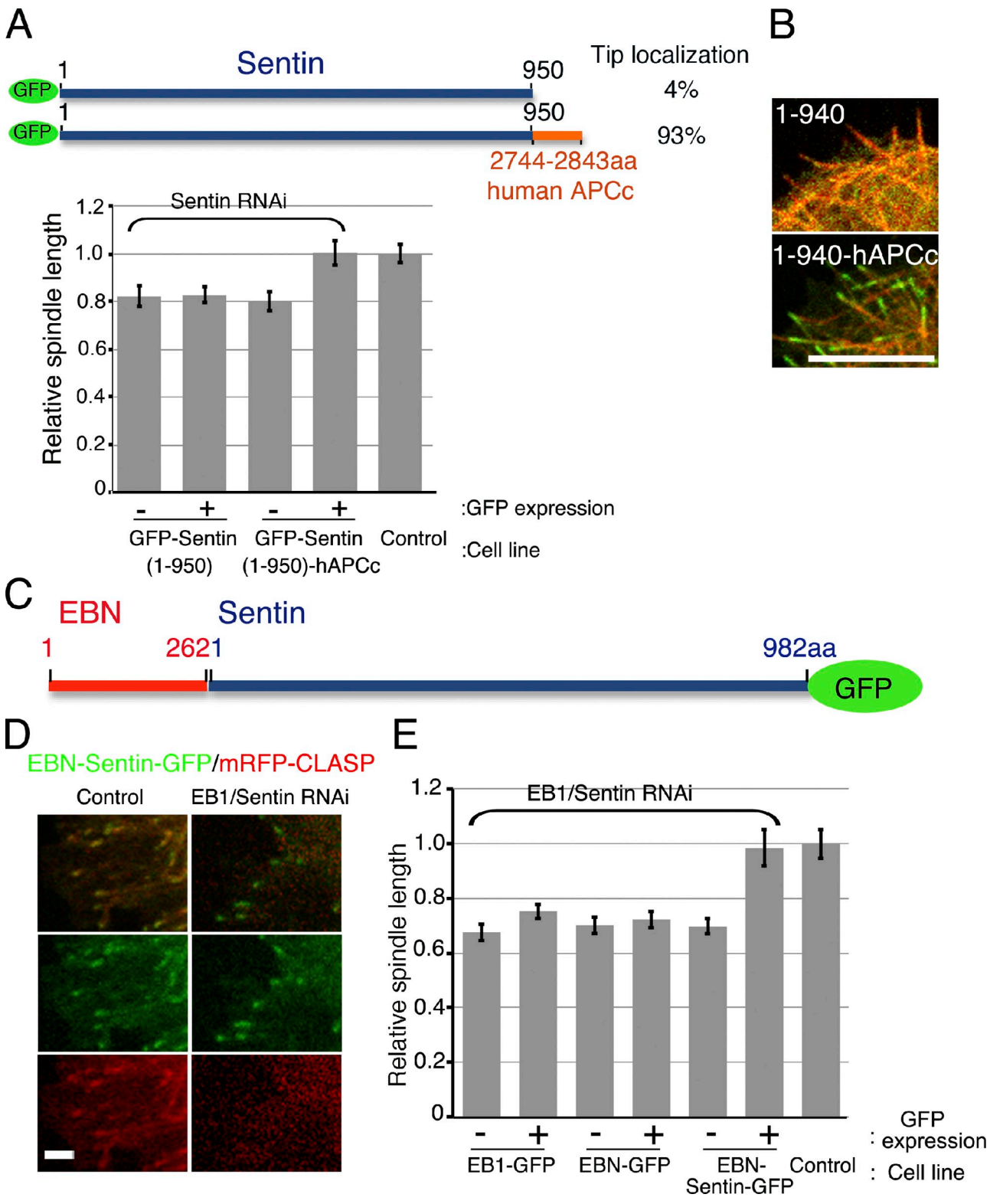

Figure 4. **Sentin recruitment to the tip restores spindle length and microtubule dynamics.** (A) The short spindle phenotype was not rescued by localization-deficient Sentin (1–950 aa) but was rescued by the plus end–tracking Sentin (1–950 aa)-hAPCc (2,744–2,843 aa) fusion gene (± SEM; *n* = 13–25). (B) Plus end–tracking ability was recovered when hAPCc was attached to GFP-Sentin (1–940 aa). Time spent in pause was also reduced by the hAPCc attachment (72 to 30%; *n* = 30). GFP is shown in green. mCherry-tubulin is shown in red. Bar, 10 µm. (C) A fusion construct in which the cargo-binding domain of EB1 (263–292 aa) is replaced by full-length Sentin-GFP. (D) EBN-Sentin-GFP and mRFP-CLASP (containing the SxIP motif) were cotransfected. EBN-Sentin-GFP, but not mRFP-CLASP, showed plus end–tracking when endogenous EB1 and Sentin were depleted, suggesting that EBN-Sentin-GFP could not interact with the SxIP motif. Also see Video 5 (another control hAPCc is also displayed). Bar, 2 µm. (E) The fusion construct EBN-Sentin-GFP, but not EB1-GFP or EBN-GFP, rescued the short spindle phenotype produced by double EB1–Sentin RNAi (± SEM; *n* = 21–26). Also see Table III and Video 5 for the interphase dynamics recovery.

Table III. **Kinetic parameters of microtubule polymerization dynamics in the EBN-Sentin-GFP rescue experiment**

Parameters	Control	EB1–Sentin RNAi	
		GFP not expressed	GFP expressed
Growth rate (μm/min)	4.5 ± 0.7	4.1 ± 0.4	4.3 ± 0.9
	4.6 ± 1.7[m]	4.0 ± 1.7[m]	4.3 ± 1.6[m]
Shrink rate (μm/min)	10.6 ± 2.3	9.6 ± 3.4	8.4 ± 0.9
	10.9 ± 5.3[m]	9.9 ± 5.9[m]	8.4 ± 3.3[m]
Catastrophe frequency (s^{-1})	0.012 ± 0.004	0.006 ± 0.004	0.016 ± 0.004
Rescue frequency (s^{-1})	0.005 ± 0.002	0.002 ± 0.002	0.010 ± 0.005
Time in growth (%)	39.3 ± 12.1	11.5 ± 7.6	36.4 ± 7.9
Time in shrink (%)	14.1 ± 4.4	5.4 ± 4.3	16.8 ± 5.3
Time in pause (%)	46.6 ± 13.2	83.1 ± 11.1	46.8 ± 11.8

Means ± SD. SD represents either microtubule to-microtubule (marked with an m) or cell to cell (others) variability (80 microtubules and 5 cells).

the EB1-binding motif of an unrelated protein (hAPCc [human APC, C-terminal 2,744–2,843 aa]) was attached to these proteins (Fig. 4, A and B). We concluded that the plus end–tracking region is necessary for the function of Sentin.

Sentin phenocopied EB1 RNAi for short bipolar spindle length and the pause-rich interphase microtubules. In contrast, our genome-wide RNAi screen and other in-depth analyses of individual proteins showed that, in S2 cells, none of the other known EB1 cargo proteins phenocopy EB1 RNAi (e.g., CLIP-190, CLASP, dynactin, and Kinesin-14; Goshima et al., 2005a, 2007; Sousa et al., 2007). These notions led us to hypothesize that Sentin might be the dominant EB1 cargo protein in S2 cells for the promotion of microtubule plus end dynamics with EB1.

To assess the function of the EB1–Sentin complex in the absence of other known EB1–cargo protein complexes, we prepared a cell line expressing at various levels the fusion gene EBN-Sentin-GFP, in which the C-terminal 30 aa of EB1 were replaced by Sentin-GFP (Fig. 4 C). Because the N-terminal microtubule-binding domain of EB1 was intact, this fusion protein was localized at the tips of growing microtubules (Fig. 4 D and Video 5). However, because the C terminus of EB1 is responsible for binding to all of the known cargo proteins (Akhmanova and Steinmetz, 2008), this fusion construct would no longer bind to other EB1 cargo proteins. Consistent with this assumption, SxIP motif-containing hAPCc-mCherry and *Drosophila* monomeric RFP (mRFP)–CLASP did not show clear plus end tracking in the presence of EBN-Sentin after knockdown of endogenous EB1 and Sentin (Fig. 4 D and Video 5). In this cell line, EBN-Sentin-GFP expression was detected for 60% of the cells (n = 500), and immunoblotting analysis indicated that the expression was lower than endogenous EB1 for the majority of the GFP-expressing cells (Fig. S3 A). Nevertheless, EBN-Sentin-GFP rescued the short spindle phenotype and the pause-rich phenotype of interphase microtubules produced by double EB1–Sentin RNAi (Figs. 4 E and S3 B, Table III, and Video 5). In contrast, EBN-GFP expression could not rescue the short spindle. Furthermore, we clonally isolated a cell line in which EBN-Sentin-GFP is expressed at the level lower than endogenous EB1 and found the rescue of the EB1–Sentin RNAi phenotype (Fig. S3, A–C). These results suggest that Sentin is the dominant cargo protein for promoting microtubule dynamics by EB1.

Plus end tracking of XMAP215^{Msps} requires Sentin

The pause-rich and short spindle phenotypes were also observed after XMAP215^{Msps} RNAi. We tested whether XMAP215^{Msps} and Sentin affect localization each other using RNAi and GFP imaging (Fig. 5). To directly assess the contribution of Sentin (or XMAP215^{Msps}) in localizing XMAP215^{Msps} (or Sentin), we selected growing microtubules in RNAi-treated cells and investigated whether their tips have punctate GFP signals (note that, albeit less frequently, microtubule growth can be seen even in the absence of XMAP215^{Msps} or Sentin). Interestingly, XMAP215^{Msps}-GFP, which tracks growing ends of microtubules in control cells (Brittle and Ohkura, 2005), did not show such localization when Sentin was knocked down by RNAi (Fig. 5 A). In contrast, EB1 or its cargo CLASP was still localized to the growing ends of microtubules after Sentin RNAi (Fig. 5 B). In the reciprocal experiment, GFP-Sentin was detected at the tip of growing microtubules both in control and XMAP215^{Msps}-depleted cells (Fig. 5 B, right). These results indicated that XMAP215^{Msps} is concentrated at the growing tips in a Sentin-dependent manner.

Sentin, a critical EB1 cargo protein for promoting microtubule dynamics

Our study identified Sentin as an EB1 cargo protein. Furthermore, a series of truncation and gene fusion experiments raised an intriguing possibility that Sentin is the dominant cargo of EB1 for microtubule dynamics promotion in S2 cells (Fig. 5 C). This idea is consistent with the fact that no other known cargo proteins phenocopy EB1 as closely as Sentin in S2 cells and that EB1 protein alone does not show growth-promoting activity in vitro in several studies, including ours (unpublished data; Bieling et al., 2007; Manna et al., 2008; Zhu et al., 2009). However, it is not ruled out that the cytoplasmic pool of other EB1 cargo proteins is also required to suppress microtubule pausing.

How might Sentin promote plus end dynamics? The first possible model is that the EB1–Sentin complex alters the structure of the plus end of microtubules, e.g., closing the sheet or promoting a specific lattice configuration, so that tubulin dimers are favorably added to or removed from the end. However, the known structural changes of the plus end can be made by the EB1 protein

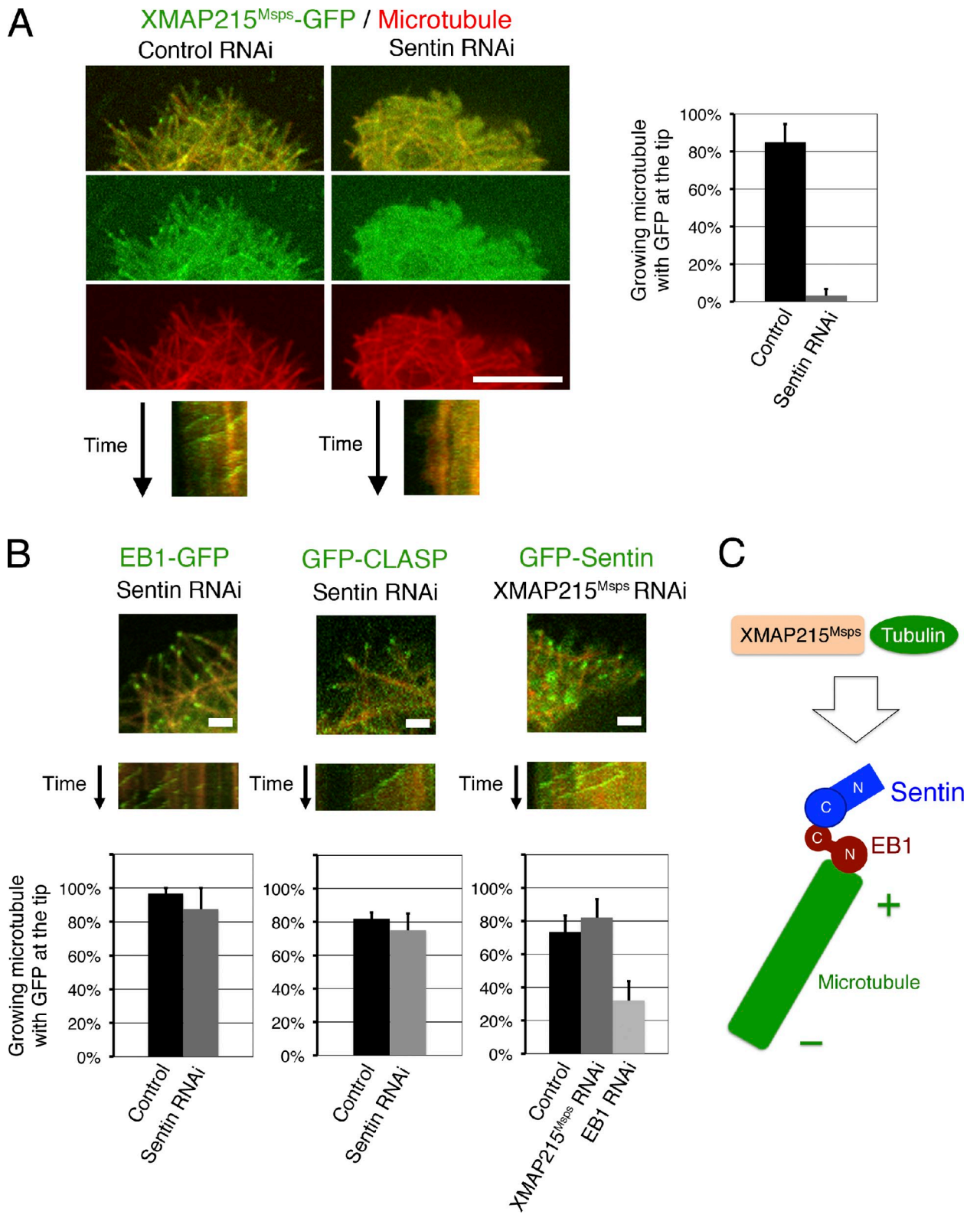

Figure 5. **XMAP215^{Msps} accumulates at the growing tip in a Sentin-dependent manner.** (A) Tip accumulation of XMAP215^{Msps} depends on Sentin. After RNAi of Sentin, XMAP215^{Msps}-GFP was scarcely detected at the plus ends of growing microtubules. Kymographs are shown at the bottom. Bar, 10 µm. Also see Video 6. (B, left and middle) EB1-GFP or GFP-CLASP localization after Sentin RNAi. Most of the growing microtubules had EB1-GFP or GFP-CLASP signals at the tips after Sentin RNAi. Kymographs are also shown. Bars, 2 µm. (right) GFP-Sentin remains at the growing tip after XMAP215^{Msps} RNAi. Error bars in each graph show SEM of six cells (each have more than five microtubules analyzed). (C) EB1- and Sentin-mediated regulation of microtubule plus end dynamics. EB1 binds to the growing ends of microtubules via its N-terminal region (N). Sentin is recruited to the plus ends by binding of its C terminus (C) to the C-terminal region of EB1. XMAP215^{Msps} and tubulin are then efficiently recruited to the plus ends of microtubules. The dissociation of tubulin from the end of the microtubules is also promoted by Sentin, which is not depicted in this cartoon.

alone (Sandblad et al., 2006; des Georges et al., 2008; Vitre et al., 2008). In the second model, Sentin catalyses the supply and removal of tubulin dimers at the microtubule plus ends, which is similar to the case of XMAP215 polymerase (Brouhard et al., 2008). These two activities may be responsible for the antipause activity of the EB1–Sentin complex observed in vivo. In the third model, EB1–Sentin makes microtubules dynamic by further recruiting XMAP215^{Msps} polymerase to the tip. This model is consistent with the phenotypic similarity among EB1, Sentin, and XMAP215^{Msps} and also the observation in *Xenopus laevis* egg extracts in which the EB1 depletion phenotype is rescued by overexpression of XMAP215 (Kronja et al., 2009). The latter two models, which are not mutually exclusive, might be experimentally tested through in vitro reconstitution of microtubule polymerization dynamics with purified EB1, Sentin, and XMAP215^{Msps}.

Materials and methods

Molecular and cell biology

The S2 expression constructs were constructed using the Gateway system (Invitrogen), and the bacterial expression constructs were made using the Gateway system or a conventional ligation reaction with pGEX or pET vectors. Cells were cultured in Schneider's medium supplemented with 10% serum. Plasmid transfection and RNAi were performed according to the previously described methods (Goshima et al., 2007; Bettencourt-Dias and Goshima, 2009; Goshima, 2010). In brief, a transfection reagent (Cellfectin; Invitrogen) was used for plasmid transfection, and stably expressing cells were selected by hygromycin. For RNAi, 1 µg of the synthesized double-stranded RNA was added to cell culture in 96-well plates. The double-stranded RNA sequences used in this study are listed in Goshima et al. (2007). The rescue experiments were performed following Goshima and Vale (2005) and Goshima (2010); 3′-UTR sequences of Sentin were amplified by PCR using the following primers: 5′-GAGCTGATCACTTCCTCC-GCCGC-3′ and 5′-TTATGGTTCGTAAGGCAAAGTTC-3′. Cdc27 RNAi was used when spindles were analyzed because this treatment significantly increases the mitotic index (Goshima et al., 2007). Stable cell lines were batch selected (no clonal isolation) or clonally isolated (Bettencourt-Dias and Goshima, 2009). For the induction of protein expression under the metallothionein promoter, the cells were cultured in the presence of 50 or 66 µM $CuSO_4$ for 3–5 d. At the end of the RNAi treatment (days 4–8), the S2 cells were resuspended, transferred to glass-bottomed concanavalin A–coated plates, and allowed to adhere for 2.5 h before fixation. Immunofluorescence microscopy for the spindle proteins was performed as previously described (Goshima et al., 2007; Bettencourt-Dias and Goshima, 2009). Methanol (EB1 and Sentin) or paraformaldehyde (others) was used for fixation. For the microtubule dynamics assays, 10 µg/ml cytochalasin D was added before imaging to eliminate the F-actin network that would constrain microtubule growth in the cytoplasm. Images were acquired at 23–25°C using a wide-field microscope (TE2000; Nikon) attached with a charge-coupled device camera (Micromax; Roper Scientific) or a spinning-disk confocal microscope (CSU-X; Yokogawa) with an EM charge-coupled device camera (ImagEM; Hamamatsu Photonics). A 100× 1.40 NA lens was used for imaging. Micromanager was used to control microscopes. Distance between two spindle poles, the regions where the microtubule minus ends are focused, was measured for evaluating spindle length in this study because some RNAi treatments, such as EB1, delocalize the centrosome relative to the spindle (Goshima et al., 2005a).

Biochemistry

Immunoprecipitation. Cell extracts were prepared by incubating the cell pellet for 20 min on ice with extraction buffer (80 mM KOH-Pipes, pH 6.8, 25 mM KCl, 4 mM $MgCl_2$, 1 mM EGTA, 1% Triton X-100, 1 mM DTT, and protease inhibitors) followed by centrifugation at 12,000 *g* for 15 min and 106,000 *g* for 15 min. The supernatant was precleared with protein A–Sepharose CL-4B beads for 1 h at 4°C. The supernatant was then incubated with anti-Sentin–conjugated protein A–Sepharose CL-4B beads for 3 h at 4°C. Beads were washed four times with extraction buffer, and bound proteins were eluted by 0.2 M glycine solution, pH 2.0. Immunoblotting was performed with anti-EB1 serum (1:1,000; gift from S. Rogers, University of North Carolina at Chapel Hill, Chapel Hill, NC) and anti-Sentin serum (1:500) or affinity-purified anti-EBN antibody (1:100).

Protein purification. GST-EB1 expression was induced in *Escherichia coli* BL21-AI with 0.2% arabinose for 16 h at 25°C. Harvested cells were lysed using the BugBuster Master Mix (EMD). After incubation with glutathione–Sepharose beads for 2 h at 4°C, GST was cleaved using the PreScission protease, and the supernatant was dialyzed using a microtubule dynamics assay buffer (MRB80 [80 mM KOH-Pipes, pH 6.8, 4 mM $MgCl_2$, and 1 mM EGTA] and 100 mM KCl) supplemented with 20% glycerol. The proteins were flash frozen in liquid nitrogen. The C-terminal fragments of Sentin (841–982 aa) tagged with 6×His and GFP were expressed in an identical manner. The supernatant after lysis was incubated with nickel-coated beads at 4°C for 1 h in the presence of 30 mM imidazole and protease inhibitors. Proteins were eluted using MRB80 containing 300 mM KCl and 200 mM imidazole followed by gel filtration using the AKTA system with a Superdex 200 10/300 GL column (GE Healthcare; equilibrated with the assay buffer containing 1 mM DTT) or the BioLogic DuoFlow system (Bio-Rad Laboratories) with the same column. The peak fraction was mixed with 20% glycerol and flash frozen in liquid nitrogen.

Pull-down assays. Full-length and truncated GST-EB1 were expressed in *E. coli* BL21-AI and attached to glutathione–Sepharose beads. After washing with PBS containing 250 mM NaCl, the beads were resuspended in PBS containing 20% glycerol and flash frozen in liquid nitrogen. For the pull-down assay using S2 extracts, 10 ml cells were lysed using 1 ml buffer containing 25 mM Tris-Cl, pH 7.4, 150 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 1% Triton X-100, and protease inhibitors for 20 min on ice. Clarified lysates were mixed with the beads associated with 100 µg GST fusion proteins for 6 h at 4°C. The beads were washed with PBS supplemented with 250 mM NaCl and resuspended with a SDS sample buffer followed by SDS-PAGE. For pull-down assays using the purified proteins, 30 µg His-GFP-Sentin (841–982 aa) was incubated with beads associated with 50 µg GST fusion protein. The binding reaction was performed in 50 µl MRB80 with 100 mM KCl and 1 mM DTT for 1.5 h at 4°C, and the beads were washed with a high salt buffer (MRB80 with 300 mM KCl and 1 mM DTT).

In vitro tracking assay. The end-tracking assay was performed as previously described (Bieling et al., 2010) with some modifications. We used commercially available poly(L-lysine)-poly(ethylene glycol)-biotin (Susos AG) and tubulin (Cytoskeleton) in MRB80. Short microtubule seeds were prepared by incubating a 50 µM tubulin mix containing 10% biotinylated tubulin and 10% rhodamine-labeled tubulin with 1 mM guanylyl α,β-methylenediphosphonate at 37°C for 20 min. Microtubule growth was initiated by flowing in 15.5 µM tubulin (containing 3.2% rhodamine-labeled tubulin) and 200 nM EB1/200 nM His-GFP-Sentin (841–982 aa) in the assay buffer (75 mM KCl, 1 mM GTP, 0.2 mg/ml κ-casein, and 0.1% methylcellulose [4000cP; Sigma-Aldrich]) and an oxygen scavenger system (50 mM glucose, 400 mg/ml glucose-oxidase, 200 mg/ml catalase, and 4 mM DTT). During the experiments, the samples were kept at 25 ± 1°C. Images were collected every 3 s using a total internal reflection fluorescence microscope (TE2000-E; Nikon) and an EM charge-coupled device camera (iXonEM+ 897; Andor) and a 488/561-nm excitation laser.

Analysis of microtubule dynamics

Because interphase microtubules occasionally swing, we manually selected a point that did move during imaging for each microtubule. We defined the distance between the point and the microtubule end as the length of each microtubule, and we generated a length-life history plot. For each astral microtubule, a kymograph was generated first to confirm that the microtubule was not bundled, and the distance between the microtubule plus ends and the centrosome was measured as the microtubule length. We defined the pause state as when the microtubule length was changed <0.3 µm for >15 s (interphase) or <0.5 µm for >5 s (aster). When microtubule growth was slower than 1 µm/min, this phase was also defined as a pause. In Fig. 5, the presence or absence of GFP comets on growing microtubules (>1 µm/min) was determined based upon kymographs.

Online supplemental material

Fig. S1 shows microtubule pausing by Sentin RNAi. Fig. S2 shows functionality and microtubule-dependent localization of GFP-Sentin. Fig. S3 shows comparison of expression levels of EBN-Sentin-GFP fusion protein and endogenous EB1. Video 1 shows interphase microtubule dynamics after control, EB1, or Sentin RNAi. Video 2 shows astral microtubule dynamics after control, Kinesin-13^{Klp10A}, or Sentin RNAi. Video 3 shows plus end tracking of GFP-Sentin. Video 4 shows plus end tracking of the C-terminal

fragment of Sentin in the presence of EB1 in vitro. Video 5 shows plus end tracking of EBN-Sentin-GFP. Video 6 shows Sentin-dependent plus end tracking of XMAP215^{Msps}-GFP. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201101108/DC1.

We are grateful to Drs. M. Dogterom and L. Laan for their help in the microtubule polymerization assay, T. Itoh (Nagoya University, Nagoya, Japan), J. Raff (University of Oxford, Oxford, England, UK), H. Ohkura (University of Edinburgh, Edinburgh, Scotland, UK), and S. Rogers for reagents, Y. Maeda, K. Matsumoto, and M. Kainosho for the use of their equipments, A. Tomida for construction of the plasmids, R. Uehara and E. Griffis for discussions, and C. Norbury for suggesting the name Sentin.

This work was supported by the Next Generation grant (Japan Society for the Promotion of Science), Grant-in-Aid for Scientific Research (Ministry of Education, Culture, Sports, Science and Technology), the Naito Foundation, the Inoue Foundation, the Uehara Foundation, the Asahi Glass Foundation, and the Human Frontier Science Program (to G. Goshima). W. Li was supported by the Global Centers of Excellence program and the State Scholarship Study Abroad Program of the Chinese Scholarship Council.

Submitted: 20 January 2011
Accepted: 10 May 2011

References

Akhmanova, A., and M.O. Steinmetz. 2008. Tracking the ends: a dynamic protein network controls the fate of microtubule tips. *Nat. Rev. Mol. Cell Biol.* 9:309–322. doi:10.1038/nrm2369

Al-Bassam, J., H. Kim, G. Brouhard, A. van Oijen, S.C. Harrison, and F. Chang. 2010. CLASP promotes microtubule rescue by recruiting tubulin dimers to the microtubule. *Dev. Cell*. 19:245–258. doi:10.1016/j.devcel.2010.07.016

Bettencourt-Dias, M., and G. Goshima. 2009. RNAi in *Drosophila* S2 cells as a tool for studying cell cycle progression. *Methods Mol. Biol.* 545:39–62. doi:10.1007/978-1-60327-993-2_3

Bettencourt-Dias, M., A. Rodrigues-Martins, L. Carpenter, M. Riparbelli, L. Lehmann, M.K. Gatt, N. Carmo, F. Balloux, G. Callaini, and D.M. Glover. 2005. SAK/PLK4 is required for centriole duplication and flagella development. *Curr. Biol.* 15:2199–2207. doi:10.1016/j.cub.2005.11.042

Bieling, P., L. Laan, H. Schek, E.L. Munteanu, L. Sandblad, M. Dogterom, D. Brunner, and T. Surrey. 2007. Reconstitution of a microtubule plus-end tracking system in vitro. *Nature*. 450:1100–1105. doi:10.1038/nature06386

Bieling, P., I.A. Telley, C. Hentrich, J. Piehler, and T. Surrey. 2010. Fluorescence microscopy assays on chemically functionalized surfaces for quantitative imaging of microtubule, motor, and +TIP dynamics. *Methods Cell Biol.* 95:555–580. doi:10.1016/S0091-679X(10)95028-0

Brittle, A.L., and H. Ohkura. 2005. Mini spindles, the XMAP215 homologue, suppresses pausing of interphase microtubules in *Drosophila*. *EMBO J.* 24:1387–1396. doi:10.1038/sj.emboj.7600629

Brouhard, G.J., J.H. Stear, T.L. Noetzel, J. Al-Bassam, K. Kinoshita, S.C. Harrison, J. Howard, and A.A. Hyman. 2008. XMAP215 is a processive microtubule polymerase. *Cell*. 132:79–88. doi:10.1016/j.cell.2007.11.043

Brunner, D., and P. Nurse. 2000. CLIP170-like tip1p spatially organizes microtubular dynamics in fission yeast. *Cell*. 102:695–704. doi:10.1016/S0092-8674(00)00091-X

Desai, A., and T.J. Mitchison. 1997. Microtubule polymerization dynamics. *Annu. Rev. Cell Dev. Biol.* 13:83–117. doi:10.1146/annurev.cellbio.13.1.83

Desai, A., S. Verma, T.J. Mitchison, and C.E. Walczak. 1999. Kin I kinesins are microtubule-destabilizing enzymes. *Cell*. 96:69–78. doi:10.1016/S0092-8674(00)80960-5

des Georges, A., M. Katsuki, D.R. Drummond, M. Osei, R.A. Cross, and L.A. Amos. 2008. Mal3, the *Schizosaccharomyces pombe* homolog of EB1, changes the microtubule lattice. *Nat. Struct. Mol. Biol.* 15:1102–1108. doi:10.1038/nsmb.1482

Dhamodharan, R., and P. Wadsworth. 1995. Modulation of microtubule dynamic instability in vivo by brain microtubule associated proteins. *J. Cell Sci.* 108:1679–1689.

Dixit, R., B. Barnett, J.E. Lazarus, M. Tokito, Y.E. Goldman, and E.L. Holzbaur. 2009. Microtubule plus-end tracking by CLIP-170 requires EB1. *Proc. Natl. Acad. Sci. USA*. 106:492–497. doi:10.1073/pnas.0807614106

Dzhindzhev, N.S., S.L. Rogers, R.D. Vale, and H. Ohkura. 2005. Distinct mechanisms govern the localisation of *Drosophila* CLIP-190 to unattached kinetochores and microtubule plus-ends. *J. Cell Sci.* 118:3781–3790. doi:10.1242/jcs.02504

Goshima, G. 2010. Assessment of mitotic spindle phenotypes in *Drosophila* S2 cells. *Methods Cell Biol.* 97:259–275. doi:10.1016/S0091-679X(10)97015-5

Goshima, G., and J.M. Scholey. 2010. Control of mitotic spindle length. *Annu. Rev. Cell Dev. Biol.* 26:21–57. doi:10.1146/annurev-cellbio-100109-104006

Goshima, G., and R.D. Vale. 2003. The roles of microtubule-based motor proteins in mitosis: comprehensive RNAi analysis in the *Drosophila* S2 cell line. *J. Cell Biol.* 162:1003–1016. doi:10.1083/jcb.200303022

Goshima, G., and R.D. Vale. 2005. Cell cycle-dependent dynamics and regulation of mitotic kinesins in *Drosophila* S2 cells. *Mol. Biol. Cell*. 16:3896–3907. doi:10.1091/mbc.E05-02-0118

Goshima, G., F. Nédélec, and R.D. Vale. 2005a. Mechanisms for focusing mitotic spindle poles by minus end–directed motor proteins. *J. Cell Biol.* 171:229–240. doi:10.1083/jcb.200505107

Goshima, G., R. Wollman, N. Stuurman, J.M. Scholey, and R.D. Vale. 2005b. Length control of the metaphase spindle. *Curr. Biol.* 15:1979–1988. doi:10.1016/j.cub.2005.09.054

Goshima, G., R. Wollman, S.S. Goodwin, N. Zhang, J.M. Scholey, R.D. Vale, and N. Stuurman. 2007. Genes required for mitotic spindle assembly in *Drosophila* S2 cells. *Science*. 316:417–421. doi:10.1126/science.1141314

Honnappa, S., S.M. Gouveia, A. Weisbrich, F.F. Damberger, N.S. Bhavesh, H. Jawhari, I. Grigoriev, F.J. van Rijssel, R.M. Buey, A. Lawera, et al. 2009. An EB1-binding motif acts as a microtubule tip localization signal. *Cell*. 138:366–376. doi:10.1016/j.cell.2009.04.065

Horio, T., and H. Hotani. 1986. Visualization of the dynamic instability of individual microtubules by dark-field microscopy. *Nature*. 321:605–607. doi:10.1038/321605a0

Howard, J., and A.A. Hyman. 2007. Microtubule polymerases and depolymerases. *Curr. Opin. Cell Biol.* 19:31–35. doi:10.1016/j.ceb.2006.12.009

Kerssemakers, J.W., E.L. Munteanu, L. Laan, T.L. Noetzel, M.E. Janson, and M. Dogterom. 2006. Assembly dynamics of microtubules at molecular resolution. *Nature*. 442:709–712. doi:10.1038/nature04928

Kinoshita, K., I. Arnal, A. Desai, D.N. Drechsel, and A.A. Hyman. 2001. Reconstitution of physiological microtubule dynamics using purified components. *Science*. 294:1340–1343. doi:10.1126/science.1064629

Komarova, Y.A., A.S. Akhmanova, S. Kojima, N. Galjart, and G.G. Borisy. 2002. Cytoplasmic linker proteins promote microtubule rescue in vivo. *J. Cell Biol.* 159:589–599. doi:10.1083/jcb.200208058

Komarova, Y., G. Lansbergen, N. Galjart, F. Grosveld, G.G. Borisy, and A. Akhmanova. 2005. EB1 and EB3 control CLIP dissociation from the ends of growing microtubules. *Mol. Biol. Cell*. 16:5334–5345. doi:10.1091/mbc.E05-07-0614

Komarova, Y., C.O. De Groot, I. Grigoriev, S.M. Gouveia, E.L. Munteanu, J.M. Schober, S. Honnappa, R.M. Buey, C.C. Hoogenraad, M. Dogterom, et al. 2009. Mammalian end binding proteins control persistent microtubule growth. *J. Cell Biol.* 184:691–706. doi:10.1083/jcb.200807179

Kronja, I., A. Kruljac-Letunic, M. Caudron-Herger, P. Bieling, and E. Karsenti. 2009. XMAP215-EB1 interaction is required for proper spindle assembly and chromosome segregation in *Xenopus* egg extract. *Mol. Biol. Cell*. 20:2684–2696. doi:10.1091/mbc.E08-10-1051

Lemos, C.L., P. Sampaio, H. Maiato, M. Costa, L.V. Omel'yanchuk, V. Liberal, and C.E. Sunkel. 2000. Mast, a conserved microtubule-associated protein required for bipolar mitotic spindle organization. *EMBO J.* 19:3668–3682. doi:10.1093/emboj/19.14.3668

Manna, T., S. Honnappa, M.O. Steinmetz, and L. Wilson. 2008. Suppression of microtubule dynamic instability by the +TIP protein EB1 and its modulation by the CAP-Gly domain of p150glued. *Biochemistry*. 47:779–786. doi:10.1021/bi701912g

Mennella, V., G.C. Rogers, S.L. Rogers, D.W. Buster, R.D. Vale, and D.J. Sharp. 2005. Functionally distinct kinesin-13 family members cooperate to regulate microtubule dynamics during interphase. *Nat. Cell Biol.* 7:235–245. doi:10.1038/ncb1222

Mitchison, T., and M. Kirschner. 1984. Dynamic instability of microtubule growth. *Nature*. 312:237–242. doi:10.1038/312237a0

Moore, A., and L. Wordeman. 2004. The mechanism, function and regulation of depolymerizing kinesins during mitosis. *Trends Cell Biol.* 14:537–546. doi:10.1016/j.tcb.2004.09.001

Moore, A.T., K.E. Rankin, G. von Dassow, L. Peris, M. Wagenbach, Y. Ovechkina, A. Andrieux, D. Job, and L. Wordeman. 2005. MCAK associates with the tips of polymerizing microtubules. *J. Cell Biol.* 169:391–397. doi:10.1083/jcb.200411089

Ohi, R., K. Burbank, Q. Liu, and T.J. Mitchison. 2007. Nonredundant functions of Kinesin-13s during meiotic spindle assembly. *Curr. Biol.* 17:953–959. doi:10.1016/j.cub.2007.04.057

Przewloka, M.R., W. Zhang, P. Costa, V. Archambault, P.P. D'Avino, K.S. Lilley, E.D. Laue, A.D. McAinsh, and D.M. Glover. 2007. Molecular analysis of

core kinetochore composition and assembly in *Drosophila melanogaster*. *PLoS ONE*. 2:e478. doi:10.1371/journal.pone.0000478

Rogers, G.C., S.L. Rogers, T.A. Schwimmer, S.C. Ems-McClung, C.E. Walczak, R.D. Vale, J.M. Scholey, and D.J. Sharp. 2004a. Two mitotic kinesins cooperate to drive sister chromatid separation during anaphase. *Nature*. 427:364–370. doi:10.1038/nature02256

Rogers, S.L., G.C. Rogers, D.J. Sharp, and R.D. Vale. 2002. *Drosophila* EB1 is important for proper assembly, dynamics, and positioning of the mitotic spindle. *J. Cell Biol.* 158:873–884. doi:10.1083/jcb.200202032

Rogers, S.L., U. Wiedemann, U. Häcker, C. Turck, and R.D. Vale. 2004b. *Drosophila* RhoGEF2 associates with microtubule plus ends in an EB1-dependent manner. *Curr. Biol.* 14:1827–1833. doi:10.1016/j.cub.2004.09.078

Sandblad, L., K.E. Busch, P. Tittmann, H. Gross, D. Brunner, and A. Hoenger. 2006. The *Schizosaccharomyces pombe* EB1 homolog Mal3p binds and stabilizes the microtubule lattice seam. *Cell*. 127:1415–1424. doi:10.1016/j.cell.2006.11.025

Slep, K.C. 2010. Structural and mechanistic insights into microtubule end-binding proteins. *Curr. Opin. Cell Biol.* 22:88–95. doi:10.1016/j.ceb.2009.10.009

Slep, K.C., S.L. Rogers, S.L. Elliott, H. Ohkura, P.A. Kolodziej, and R.D. Vale. 2005. Structural determinants for EB1-mediated recruitment of APC and spectraplakins to the microtubule plus end. *J. Cell Biol.* 168:587–598. doi:10.1083/jcb.200410114

Sousa, A., R. Reis, P. Sampaio, and C.E. Sunkel. 2007. The *Drosophila* CLASP homologue, Mast/Orbit regulates the dynamic behaviour of interphase microtubules by promoting the pause state. *Cell Motil. Cytoskeleton*. 64:605–620. doi:10.1002/cm.20208

Stevens, N.R., J. Dobbelaere, K. Brunk, A. Franz, and J.W. Raff. 2010. *Drosophila* Ana2 is a conserved centriole duplication factor. *J. Cell Biol.* 188:313–323. doi:10.1083/jcb.200910016

Tirnauer, J.S., S. Grego, E.D. Salmon, and T.J. Mitchison. 2002. EB1-microtubule interactions in *Xenopus* egg extracts: role of EB1 in microtubule stabilization and mechanisms of targeting to microtubules. *Mol. Biol. Cell*. 13:3614–3626. doi:10.1091/mbc.02-04-0210

Uehara, R., R.S. Nozawa, A. Tomioka, S. Petry, R.D. Vale, C. Obuse, and G. Goshima. 2009. The augmin complex plays a critical role in spindle microtubule generation for mitotic progression and cytokinesis in human cells. *Proc. Natl. Acad. Sci. USA*. 106:6998–7003. doi:10.1073/pnas.0901587106

Vitre, B., F.M. Coquelle, C. Heichette, C. Garnier, D. Chrétien, and I. Arnal. 2008. EB1 regulates microtubule dynamics and tubulin sheet closure in vitro. *Nat. Cell Biol.* 10:415–421. doi:10.1038/ncb1703

Yao, M., Y. Wakamatsu, T.J. Itoh, T. Shoji, and T. Hashimoto. 2008. Arabidopsis SPIRAL2 promotes uninterrupted microtubule growth by suppressing the pause state of microtubule dynamics. *J. Cell Sci.* 121:2372–2381. doi:10.1242/jcs.030221

Zhu, Z.C., K.K. Gupta, A.R. Slabbekoorn, B.A. Paulson, E.S. Folker, and H.V. Goodson. 2009. Interactions between EB1 and microtubules: dramatic effect of affinity tags and evidence for cooperative behavior. *J. Biol. Chem.* 284:32651–32661. doi:10.1074/jbc.M109.013466