EB1 promotes microtubule dynamics by recruiting Sentin in Drosophila cells

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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 (polym-
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 reconstructed 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 (Sousa et al., 2007). 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; 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 co-knockdown of two, but not all three, EB1 family members in mammals (Komarova et al., 2009). In S2 cells, EB1 or XMAP215Msps RNAi knockdown increases the pause during interphase and results in the formation of shorter spindles (Rogers et al., 2002; Brittle and Ohkura, 2005). Kinesin-13Klp10A 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 XMAP215Msps 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 XMAP215Msps 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 XMAP215Msps-depleted cells (Rogers et al., 2002; Rogers et al., 2002; Brittle and Ohkura, 2005), Kinesin-13Klp10A 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 XMAP215Msps accumulation at the growing microtubule ends and, thus, provide a new insight into the mechanism of EB1-dependent regulation of microtubule plus ends.

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

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>EB1</th>
<th>Sentin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth rate (µm/min)</td>
<td>5.3 ± 1.4</td>
<td>4.5 ± 0.8</td>
<td>3.5 ± 0.5</td>
</tr>
<tr>
<td>Shrink rate (µm/min)</td>
<td>11.3 ± 1.9</td>
<td>7.9 ± 1.4</td>
<td>7.7 ± 2.0</td>
</tr>
<tr>
<td>Catastrophe frequency [s⁻¹]</td>
<td>0.011 ± 0.003</td>
<td>0.005 ± 0.001</td>
<td>0.005 ± 0.001</td>
</tr>
<tr>
<td>Time in growth (%)</td>
<td>39.0 ± 11.5</td>
<td>14.1 ± 8.2</td>
<td>20.8 ± 11.1</td>
</tr>
<tr>
<td>Time in shrink (%)</td>
<td>19.7 ± 5.0</td>
<td>7.9 ± 3.1</td>
<td>8.0 ± 1.6</td>
</tr>
<tr>
<td>Time in pause (%)</td>
<td>41.3 ± 10.5</td>
<td>78.0 ± 10.8</td>
<td>71.2 ± 11.7</td>
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</tbody>
</table>

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.

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

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Sentin</th>
<th>Kinesin-13Klp10A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth rate (µm/min)</td>
<td>10.8 ± 1.6</td>
<td>8.8 ± 2.4</td>
<td>9.8 ± 1.3</td>
</tr>
<tr>
<td>Shrink rate (µm/min)</td>
<td>19.6 ± 4.4</td>
<td>21.6 ± 4.9</td>
<td>40.2 ± 8.7</td>
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<tr>
<td>Catastrophe frequency [s⁻¹]</td>
<td>0.035 ± 0.006</td>
<td>0.021 ± 0.008</td>
<td>0.025 ± 0.007</td>
</tr>
<tr>
<td>Time in growth (%)</td>
<td>53.1 ± 8.6</td>
<td>34.7 ± 15.3</td>
<td>62.7 ± 8.4</td>
</tr>
<tr>
<td>Time in shrink (%)</td>
<td>32.0 ± 7.5</td>
<td>17.8 ± 7.3</td>
<td>18.9 ± 6.0</td>
</tr>
<tr>
<td>Time in pause (%)</td>
<td>14.9 ± 11.1</td>
<td>47.5 ± 17.7</td>
<td>18.4 ± 10.6</td>
</tr>
</tbody>
</table>

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.
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 aa) tagged with GFP and showed its direct binding to GST-EB1 (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 (red) in the in vitro plus end–tracking assay, Bar, 5 µm. Also see Video 4.

**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.
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.
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 XMAP215Msps requires Sentin

The pause-rich and short spindle phenotypes were also observed after XMAP215Msps RNAi. We tested whether XMAP215Msps and Sentin affect localization each other using RNAi and GFP imaging (Fig. 5). To directly assess the contribution of Sentin (or XMAP215Msps) in localizing XMAP215Msps (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 XMAP215Msps or Sentin). Interestingly, XMAP215Msps-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 XMAP215Msps-depleted cells (Fig. 5 B, right). These results indicated that XMAP215Msps 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. XMAP215Msps accumulates at the growing tip in a Sentin-dependent manner. (A) Tip accumulation of XMAP215Msps depends on Sentin. After RNAi of Sentin, XMAP215Msps-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 XMAP215Msps 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. XMAP215Msps 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 catalyzes 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 microtubule dynamics by further recruiting XMAP215<sup>Msps</sup> polymerase to the tip. This model is consistent with the phenotypic similarity among EB1, Sentin, and XMAP215<sup>Msps</sup> 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<sup>Msps</sup>.

**Materials and methods**

**Molecular and cell biology**

The 52 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′-GAGCGTACATCCTTCCGCGCCG-3′ and 5′-TTATGGTGCAAGGCAGAATGTCC-3′.

Cdc27 RNAi was used when spindles were analyzed because this treatment significantly increased 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 500 µM ZnCl<sub>2</sub> and 1 µg/ml puromycin (Invitrogen), and the bacterial expression constructs were made using the Gateway system (Invitrogen).

**Biochemistry**

**Immunoprecipitation.** Cell extracts were prepared by incubating the cell pellet (200 µl, 0.5–1 × 10<sup>6</sup> cells) with extraction buffer (80 mM KOH-Pipes, pH 6.8, 1 mM EDTA, and protease inhibitors) followed by centrifugation at 12,000 × g for 15 min. The supernatant was pre-cleared with protein A-Sepharose CL-4B beads for 2 h at 4°C. The supernatant was then incubated with anti-Sentin–conjugated protein A-Sepharose CL-4B beads for 2 h at 4°C. Beads were washed four times with extraction buffer, and bound proteins were eluted with 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-EB1 antibody (1:100).

**Protein purification.** GST-EB1 expression was induced in *E. 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<sub>2</sub>, 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 (B41–982 aa) tagged with 6×His and GFP were expressed in an identical manner. The supernatant after lysis was incubated with nickel-coupled beads at 4°C for 1 h in the presence of 30 mM imidazole and protease inhibitors. Proteins were eluted using MRB80 containing 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 52 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 (B41–982 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 polyethylene glycol-biotin (Sasos AG) and tubulin (Cytoskeleton) in MRB80. Short microtubule seeds were prepared by incubating a 30 µM tubulin mix containing 10% biotinylated tubulin and 10% rhodamine-labeled tubulin with 1 µM guanosyl γ, β-methylene phosphonate at 37°C for 20 min. Microtubule growth was initiated by flow- ing in 15.5 µM tubulin (containing 3% rhodamine-labeled tubulin) and 200 nM EB1/200 nM His-GFP-Sentin (B41–982 aa) in the assay buffer (75 mM KCl, 1 mM GTP, 0.2 mg/ml x-casein, and 0.1% methylcellulose [400000 cP; 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 (TE2000E; 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-time 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<sup>↑↑</sup>/Eg5 RNAi. Video 3 shows plus end tracking of GFP-Sentin. Video 4 shows end tracking of the C-terminal.
fragment of Sentinel in the presence of EB1 in vitro. Video 5 shows plus end tracking of EB1-Sentin-GFP. Video 6 shows Sentin-dependent plus end tracking of XMAP215-GFP. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.20110108/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. Boff (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. Kaino for the use of their equipments, A. Tomida for construction of the plasmids, R. Uehara and E. Griﬃths for discussions, and C. Norbury for suggesting the name Sentinel.

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


Figure S1. **Microtubule pausing after Sentin RNAi.** (A) Immunoblots showing the efficient knockdown of Sentin and EB1. Our quantification indicated that Sentin is depleted to 5–10% and EB1 to 10–20% compared with control cells. Double RNAi knockdown was also as efficient. (B) Tracking of interphase microtubule plus ends that were far (>5 µm) from the cell cortex. The plus end of a microtubule is marked with white circles, and the cell edges are shown with white lines. (bottom) Life history plots of each of the three microtubules of control or Sentin RNAi cells. Bar, 5 µm.
Figure S2. **GFP-Sentin is functional and localized at the growing microtubule tip.** (A) GFP-Sentin rescued the short metaphase spindle phenotype produced by RNAi against the 3′-UTR region of Sentin (+ SEM; \( n = 16-27 \)) that specifically reduced endogenous Sentin, indicating that this fusion gene is functional. (B) Diffused distribution of GFP-Sentin (green) in the absence of microtubules (+colcemid). Chromosomes were stained with Hoechst 33342 (blue). Bar, 5 \( \mu \)m.
Figure S3. **EBN-Sentin-GFP rescued double EB1–Sentin RNAi at the expression level lower than endogenous EB1.** (A) Comparison of expression levels of endogenous EB1 and ectopically expressed EBN-Sentin-GFP by immunoblotting using the anti-EBN antibody. (left) EB1 antisera were affinity purified using a recombinant EBN fragment (1–208 aa). Immunoblot and Coomassie staining for recombinant proteins demonstrate that the purified anti-EBN antibody specifically recognizes the N-terminal region of EB1. (middle) The extract of the batch-selected S2 cell line expressing EBN-Sentin-GFP was immunoblotted with the anti-EBN antibody. The band corresponding to EBN-Sentin-GFP was 16% in intensity compared with endogenous EB1. (right) The extract of an EBN-Sentin-GFP clone line was immunoblotted with anti-EBN antibody. The intensity ratio of EBN-Sentin-GFP relative to endogenous EB1 was 4%. Lower expression of EBN-Sentin-GFP than endogenous EB1 was also confirmed by comparative immunoblotting of these proteins using S2 cell extracts and the recombinants (not depicted). FL, full length; N, N-terminal; C, C-terminal. (B) EBN-Sentin-GFP expression level and metaphase spindle length in the batch-selected cell line. The mean signal intensity of EBN-Sentin-GFP in the whole cell after control (left) or endogenous EB1–Sentin knockdown (right) was measured for each cell. Blue dots correspond to those cells in which GFP signals were not recognized by eyes, whereas red dots were used when GFP signals were detected. Most of the cells expressing EBN-Sentin-GFP had the spindle length >7.2 µm (mean of EB1–Sentin double RNAi samples without detectable GFP expression). In the clonal line, the mean intensity of GFP signals was 60 arbitrary units (AU). (C) Rescue of the spindle length phenotype of EB1 RNAi by lower than endogenous expression of EBN-Sentin-GFP (± SEM; n = 4–16). Spindle length was restored when EBN-Sentin-GFP was weakly expressed in the clone line.
Video 1. **Interphase microtubule dynamics in Drosophila S2 cells after control, EB1, or Sentin RNAi.** Cells expressing GFP-tubulin were imaged using a wide-field microscope (TE2000). Microtubules were less dynamic in the absence of EB1 or Sentin. 15 frames per second (fps); 45x real speed. Bar, 2 µm.

Video 2. **Astral microtubule dynamics in Drosophila S2 cells after control, Kinesin-13<sup>Klp10A</sup>, or Sentin RNAi.** Cells expressing GFP-tubulin were imaged using a spinning-disk confocal microscope. Microtubules were less dynamic in the absence of Sentin. 30 fps; 30x real speed. Bar, 5 µm.

Video 3. **Plus end tracking of GFP-Sentin.** GFP-Sentin (green) was localized to the growing ends of microtubules (red) during interphase (left) and mitosis (right). Images were taken using a spinning-disk confocal microscope. 30 fps; 90x real speed. Bars, 10 µm.

Video 4. **Plus end tracking of the C-terminal fragment of Sentin in the presence of EB1 in vitro.** The C-terminal fragment of Sentin (841–982 aa) tagged with GFP (green) tracked the growing end of microtubules in the presence of EB1 in vitro. Images were acquired using a total internal reflection fluorescence microscope. 20 fps; 60x real speed. Bar, 2 µm.

Video 5. **Plus end tracking of EBN-Sentin-GFP.** (left) Plus end tracking of EBN-Sentin-GFP rescued the interphase microtubule dynamics. Endogenous EB1 and Sentin were depleted by RNAi. (right) EBN-Sentin-GFP could not recruit Drosophila CLASP or hAPC<sub>c</sub> to the growing microtubule ends when endogenous EB1 and Sentin were depleted. Images were acquired by a spinning-disk confocal microscope. 15 fps; 45x real speed. Bar, 5 µm.

Video 6. **Sentin-dependent plus end tracking of XMAP215<sup>Mspo</sup>-GFP.** XMAP215<sup>Mspo</sup>-GFP (middle, green) accumulates at the tip of growing microtubules (bottom, red) in a Sentin-dependent manner. XMAP215<sup>Mspo</sup>-GFP and mCherry-tubulin were transiently expressed along with indicated RNAi treatment. Images were acquired by a spinning-disk confocal microscope. 30 fps; 90x real speed. Bar, 10 µm.