Single-particle imaging reveals intraflagellar transport–independent transport and accumulation of EB1 in *Chlamydomonas* flagella

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ABSTRACT The microtubule (MT) plus-end tracking protein EB1 is present at the tips of cilia and flagella; end-binding protein 1 (EB1) remains at the tip during flagellar shortening and in the absence of intraflagellar transport (IFT), the predominant protein transport system in flagella. To investigate how EB1 accumulates at the flagellar tip, we used in vivo imaging of fluorescent protein–tagged EB1 (EB1-FP) in Chlamydomonas reinhardtii. After photobleaching, the EB1 signal at the flagellar tip recovered within minutes, indicating an exchange with unbleached EB1 entering the flagella from the cell body. EB1 moved independent of IFT trains, and EB1-FP recovery did not require the IFT pathway. Single-particle imaging showed that EB1-FP is highly mobile along the flagellar shaft and displays a markedly reduced mobility near the flagellar tip. Individual EB1-FP particles dwelled for several seconds near the flagellar tip, suggesting the presence of stable EB1 binding sites. In simulations, the two distinct phases of EB1 mobility are sufficient to explain its accumulation at the tip. We propose that proteins uniformly distributed throughout the cytoplasm like EB1 accumulate locally by diffusion and capture; IFT, in contrast, might be required to transport proteins against cellular concentration gradients into or out of cilia.

INTRODUCTION

Microtubules (MTs) are polar assemblies of α- and β-tubulin. The MT plus-end is more dynamic, and various proteins bind to the plus-end, promoting MT elongation or shortening (Akhmanova and Steinmetz, 2010). End-binding protein 1 (EB1) is a widely distributed plus-end tracking protein that binds directly to MTs in vitro. EB1 has been widely used to track the tips of growing MTs and as an indicator for the presence of GTP/GDP+Pi tubulin near the plus-end. While polymerized GTP and GDP+Pi tubulin are its preferred targets, EB1 also binds to more subdistal regions of growing MTs, suggesting that its binding is not strictly coupled to the nucleotide state of tubulin but is also sensitive to the conformational state of tubulin in the MT lattice (Maurer et al., 2011).

The plus-ends of the axonemal microtubules are at the distal tips of cilium and flagella. At the tip reside several tip-binding proteins, including kinesin-13 (Piao et al., 2009; Vasudevan et al., 2015), kinesin-4/Kif-7 (He et al., 2014), Che-12/crescerin (Das et al., 2015), Cep104/FAP256 (Satish Tammana et al., 2013), and Spef1/CLAMP (Gray et al., 2009). Similarly, EB proteins have been shown to be present at the tips of motile 9+2 flagella, mammalian primary cilia, and sensory cilia in Caenorhabditis elegans (Pedersen et al., 2003; Hao et al., 2011; Schroder et al., 2011). These results reveal that, within both motile and primary cilium, EB1 accumulates on the tips of apparently static axonemal MTs in contrast with its behavior on singlet MTs in the cytoplasm, where EB1 binding is largely limited to the plus-ends of growing MTs. In Chlamydomonas reinhardtii, EB1 remains at the tips of steady-state and even shortening flagella (Pedersen et al., 2003). Axonemal MTs are distinct from singlet MTs in the cell body due to their high content of posttranslationally modified tubulin and their dense decoration with associated structures, including integral protein ribbons (Linck et al., 2014). Further, the A-tubules of the outer doublets and the central-pair singlet MTs are...
capped by material of largely unknown composition (Dentler and Rosenbaum, 1977; Satish Tamman et al., 2013). These biochemical and ultrastructural specializations might be the reason for the resistance to MT-depolymerizing drugs, the high biochemical stability, and the apparent lack of treadmilling and dynamic instability of the axonemal MTs (Marshall and Rosenbaum, 2001; Watanabe et al., 2004). However, a continual albeit low-level incorporation of tubulin was demonstrated for steady-state flagella of zygotes (Marshall and Rosenbaum, 2001). These observations raise the question of whether EB1 binding to the flagellar tip is similarly transient and dependent on the addition of fresh tubulin as described for its binding to cytoplasmic singlet MTs.

Many flagellar proteins require intraflagellar transport (IFT), a motor-driven bidirectional motility of proteins along the axonemal MTs, to efficiently enter flagella and move to the tip (Rosenbaum and Witman, 2002; Wren et al., 2013; Craft et al., 2015). Pedersen et al. (2003) showed that EB1 remains at the tips of flagella after IFT has been switched off in Chlamydomonas fla10-1, a temperature-sensitive mutant in the anterograde IFT motor kinesin-2 (Kozminski et al., 1995). Taking into account that EB1 remains present at the flagellar tips of nongrowing flagella, these observations raise the possibility that EB1 is firmly attached to the flagellar tip and, once deposited at the tip via IFT, remains in that location in the absence of IFT. Alternatively, EB1 at the tip could be continuously exchanged by an IFT-independent mechanism.

In this study, we used in vivo imaging in C. reinhardtii to elucidate the dynamics of fluorescent protein (FP)-tagged EB1 in flagella. The FP tag (green fluorescent protein [GFP] or mNeonGreen [NG]) was fused to the C-terminus of EB1; such fusions are thought to be neutral with respect to EB1 dimerization and microtubule plus-end tracking but do interfere with the binding of some EB1-interacting proteins (Skube et al., 2010; Sen et al., 2013). C. reinhardtii tends to adhere to its two flagella to a cover glass, allowing for the tracking of single fluorescent particles in flagella by total internal reflection fluorescence (TIRF) microscopy (Lechtrek, 2013). IFT transport of EB1-FP was essentially absent and EB1-FP entered flagella by diffusion and dwelled transiently at the tip. In simulations, these two distinct phases of EB1 mobility in flagella were sufficient to explain its accumulation at the tip. Our data show that proteins can rapidly accumulate at the flagellar tip in an IFT-independent manner.

RESULTS

EB1 and EB1-GFP show a similar subcellular distribution

Antibody staining showed that EB1 is present in the cell bodies and at the flagellar tips of C. reinhardtii (Pedersen et al., 2003). To visualize the in vivo dynamics of EB1, we expressed EB1 fused to either GFP or the brighter NG in wild-type cells (Figure 1A; Shaner et al., 2004). Alternatively, EB1 at the flagellar tip is continuously exchanged with the hitherto unknown dynamics of the cortical MTs in C. reinhardtii (arrowheads in Figure 2A; Supplemental Movie S1). Kymograms were used to determine the velocity of EB1-NG comets (Figure 2B); the average velocity was 0.142 μm/s (±0.04 μm/s, n = 31; Figure 2C). This corresponds to a rate of ~8.5 μm/min, which is within the range determined for MT growth in plant (~5 μm/min) and mammalian cells (~10–20 μm/min; Mimori-Kiyosue et al., 2000; Chan et al., 2003; Salaycik et al., 2005). Near the posterior end of the cell, most EB1-NG comets became slower and weaker, but some reached the edge of the cell and continued to grow in a curve along the edge before the comet was lost. After the comets vanished, some EB1-NG remained attached to the MT and, occasionally, we observed rapid shrinkage of these signals progressing from the posterior cell region toward the anterior, indicating catastrophic depolymerization of the underlying MT (Figure 2D). We conclude that a subset of the cortical MTs of C. reinhardtii is highly dynamic and that its flagellar basal apparatus continuously nucleates MTs, similar to the mammalian centrosome. The data further establish that C. reinhardtii EB1-NG behaves similarly to EB1 in other organisms in tracking the plus-ends of growing MTs.

EB1 at the flagellar tip is exchanged in an IFT-independent manner

Previous data have shown that EB1 remains at the flagellar tip under various experimental conditions, including flagella shortening and inhibition of IFT. These observations could indicate that EB1 is firmly bound to the flagellar tip. To test the dynamics of flagellar EB1, we utilized fluorescence recovery after photobleaching (FRAP) analysis after bleaching either the entire flagellum (Supplemental Figure S1) or only the flagellar tip (Figure 3A; Supplemental Movie S2); both methods gave very similar results. Fluorescence recovery at the flagellar tip was apparent briefly after photobleaching, and full recovery of fluorescence was achieved in 3–7 min (Figure 3, B and C). Complete or near-complete recovery was also observed after repeated bleaching of the flagellar tip (Supplemental Figure S1). In conclusion, EB1 at the flagellar tip is continuously exchanged with unbleached protein from the flagellum and the cell body.
EB1 diffusion in flagella

IFT is thought to transport most flagellar proteins into and inside cilia and flagella. In C. reinhardtii, transport by IFT has been demonstrated for various axonemal proteins (Wren et al., 2013; Craft et al., 2015). To determine whether EB1 translocation to the tip and thus the recovery of EB1-FP fluorescence was IFT-dependent, we expressed EB1-FP in fla10-1, which allows one to switch off IFT by incubating cells at elevated temperatures. Because many cells moved during extended experiments, the recovery rate (percent recovery of the prebleach signal intensity/minute) instead of total recovery time was used to compare EB1-FP recovery at different conditions (Figure 3, E and F). FRAP analysis showed that there was no significant difference in the rate of EB1-FP fluorescence recovery between fla10-1 cells maintained at the permissive (22°C) or restrictive (32°C) temperature (Figure 3, D and F). Western blotting confirmed that the temperature shift was effective in abolishing IFT: at the restrictive temperature selected, IFT particle proteins were quantitatively

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**FIGURE 1:** The cellular distribution of endogenous and FP-tagged EB1 is similar. (A) Schematic presentation of the EB1-FP expression vector. The sequence for either GFP or NG were integrated into the genomic DNA encompassing the EB1 gene, including its endogenous promoter (pro) and terminator (term). The selectable marker gene aphVIII was present on the same plasmid. The arrows indicate the orientation of the genes. (B) Western blot analysis of whole cells and isolated flagella of wild type (control) and strains expressing EB1-GFP or EB1-NG probed with antibodies to EB1, and α-tubulin. The flagellar samples were 70 times more concentrated than the whole-cell samples (i.e., ~140 flagella/cell). (C) Flagellar extracts from a EB1-GFP–expressing strain and a wild-type control were incubated with anti-GFP beads and the depleted extract (Unbound), the bound fraction (Eluate), and the original extract (Input), were analyzed by SDS–PAGE and Western blotting with anti-EB1. Note that endogenous EB1 copurifies with EB1-GFP. (D) Silver staining of the eluate obtained from a strain expressing EB1-GFP by GFP affinity purification. (E) DIC (a), TIRF (b), and the corresponding merged image (c) of live EB1-GFP cells. Arrowheads in a, flagellar tips; arrows in d, punctae of EB1-NG in a posterior region of the cell. Scale bar: 3 μm. (F) Schematic representation (left) and live images of a focal series through a EB1-NG cell. Arrowheads in a, flagellar tips; arrows in d, punctae of EB1-NG in a posterior region of the cell. Scale bar: 3 μm.
removed from fla10-1 flagella; the levels of endogenous EB1 remained constant (Supplemental Figure S2C). Next we tested FRAP of EB1-NG at the flagellar tip of fla11-1, a temperature-sensitive mutant defective in the IFT protein IFT172, which is thought to interact with EB1 (Pedersen et al., 2005). Previous reports using antibodies did not observe EB1 at the flagellar tips of fla11-1 mutants maintained at the restrictive temperature (Pedersen et al., 2003). However, TIRF microscopy revealed that EB1-FP was present at the flagellar tips and recovered normally after photobleaching in fla11-1 cells maintained at 22°C and 32°C (Supplemental Figure S2, A and B). Western blotting of flagellar preparations from fla11-1 showed that the levels of endogenous EB1 were unaffected by the temperature shift, while the levels of IFT172 were strongly reduced (Supplemental Figure S2C). We noticed that, at the permissive temperature and even more at the restrictive temperature, many fla11-1 flagella were rather short and accumulated IFT proteins at the tips (unpublished data), which could mask EB1 detection by antibodies in immunofluorescence and distort protein ratios in Western blots, putatively explaining previous findings describing the absence of EB1 from fla11-1 flagella at the restrictive temperature.

Two-color imaging revealed that EB1-NG and the IFT particle protein IFT20-mCherry move independently from each other, with IFT20 moving by IFT and EB1-FP moving by diffusion (Figure 3G). EB1-NG also moved independently of the bona fide IFT cargo mCherry-α-tubulin in steady-state and growing flagella (Supplemental Figure S3). For the entire duration of this study, transport of EB1-FP by IFT was observed only once. IFT transport of GFP-tagged EB1 and EB3 proteins was also not apparent in primary cilia of retinal pigment epithelium cells or C. elegans sensory cilia (Hao et al., 2011; Larsen et al., 2013). The data indicate that EB1-FP enters flagella and translocates to the tips independently of IFT.

Axonemal MTs bind less EB1-NG than cytoplasmic MTs
Similar to observations in other systems, C. reinhardtii EB1-NG preferably binds to the end of growing cell body MTs and vanishes from nongrowing MTs. However, EB1 is present at the tips of steady-state, growing, and even shrinking flagella (Pedersen et al., 2003), raising the question of whether the mechanisms of EB1 binding to axonemal and cytoplasmic MTs are different. We compared the intensity of the EB1-NG signal at the tips of steady-state flagella with that of the EB1-NG comets in the cell body. The latter is likely to represent the tip of a single MT, while the former contains nine A- and nine B-tubules and two central-pair MTs. The fluorescence intensity of one EB1-NG comet typically exceeded that of the total EB1-NG present at the flagellar tip (478 vs. 257 a.u. for comets and flagellar tips; SD 89.9, n = 4 and SD 74.0, n = 10, respectively). To test whether EB1-NG might be predominantly attached to the two CP MTs, we expressed EB1-NG in the central pair (CP)-deficient mutant pf18; the amounts and dynamics of EB1-NG

![Figure 2](https://example.com/figure2.png)

**Figure 2:** Fluorescent EB1 localizes to comets in the cell body. (A and B) Individual frames (A) and corresponding kymogram (B) from a recording of EB1-NG comets in the cell body. The comet marked by arrowheads moved from the flagella-bearing cell apex to its posterior, presumably tracking the tip of an elongating MT. Scale bar: 2 μm. (B) Kymogram of the comet marked in A. Dashed lines indicate time points corresponding to the frames in A. Arrowheads with A and P, anterior and posterior of the cell. Scale bar: 1 μm and 5 s. (C) Histogram depicting the distribution of the velocities of EB1-NG comets. (D, a–e) Single frames from a video depicting EB1-NG loss, presumably during catastrophic MT shortening. Scale bar: 2 μm. (f) Kymogram corresponding to a; the arrow indicates the trace corresponding to the EB1 signal labeled in a. (g) Kymogram showing growth and retreat of an EB1-NG comet. Arrowhead, elongation; arrows, catastrophe. Scale bars: 1 μm and 10 s. See Supplemental Movie S1.
at the tips of pf18 flagella were essentially unaltered (unpublished data). In conclusion, only small amounts of EB1 are present at the flagellar tip, suggesting that the plus-ends of axonemal MTs in steady-state flagella attract considerably less EB1 than the tips of growing singlet MTs in the cell body.

For determining whether the growth state of flagella affects the amount of EB1-NG at the tip, cells were deflagellated by a pH shock and allowed to initiate flagellar regeneration. Cells with regenerating and steady-state flagella were mixed before imaging to allow for a direct comparison of signal strengths (Figure 4A). The EB1-NG signal at the tips of regenerating flagella was on average 2.5 times brighter than that of steady-state flagella (Figure 4B) and often extended into the flagellar shaft. FRAP analysis of growing and steady-state flagella showed similar rates of EB1-NG exchange (Supplemental Figure S2D). Using mechanical shearing, we generated cells with only one flagellum and analyzed EB1-NG distribution while such long-zero cells regrew the missing flagellum and shortened the remaining flagellum (Rosenbaum et al., 1969). EB1-NG remained attached to the tips of the longer, putatively retracting flagella, and the EB1-NG signals at the tips of growing flagella of such long-short cells were increased ~twofold in strength, often extending into the flagellar shaft (Figure 4, C and D). These single-cell experiments show that the tips of flagella with elongating axonemes have an increased capacity to attract EB1-FP.

FIGURE 3: EB1 at the flagellar tip is rapidly exchanged independent of/unaids by IFT. (A and B) Individual frames (A) and corresponding kymogram (B) from a FRAP experiment demonstrating the exchange of EB1-NG at the tips of steady-state flagella. (A) Images taken before (pre) and at various time points (0–320 s) after photobleaching of the flagellar tip using a spot laser (position indicated by the dashed red circle). The dashed white box indicates the area used for FRAP analysis. In the kymogram (B), the flagellar tip and base and the bleaching step are indicated. Scale bars: 1 μm and 20 s. (C) Quantitative analysis of a FRAP experiment. The recovery of fluorescence (in arbitrary units, a.u.) at the flagellar tip was measured after photobleaching of the entire flagellum. The signal recovers to prebleach strength in ~3 min. Arrowhead, bleaching step. (D) Kymograms depicting recovery of EB1-GFP in flagella of fla10-1 cells maintained at 22°C and 32°C. The base of the flagella (B) and the distal tip (T) are marked. Scale bars: 2 μm and 10 s. (E and F) Mean recovery rates of wild-type (E) and fla10-1 (F) cells expressing EB1-NG and EB1-GFP, respectively. Cells were analyzed at the permissive (22°C) and restrictive (32°C) temperatures for IFT in fla10-1. Error bars indicate the SD. The differences in the rates of fla10-1 and the control strain are likely to be caused by differences in the microscope settings. (G) Merged kymogram from simultaneous imaging of EB1-NG (green) and IFT20-mCherry (red) in flagella. Note that EB1 and IFT20 move independent of each other. The base of the flagellum (base) and the distal tip (tip) are marked. Scale bars: 2 μm and 10 s.
Limited turnover of axonemal tubulin in steady-state flagella appears unrelated to EB1 binding

The increased presence of EB1-NG at the tips of elongating flagella suggests a causal link between tubulin polymerization and EB1-NG binding to axonemal MTs, raising the question of whether EB1 accumulation at the tips of steady-state and shortening flagella also depends on the addition of new tubulin to the axonemal MTs. Treadmilling of axonemal MTs has not been observed, and flagellar length is essentially constant within the short periods required for EB1-FP recovery (Marshall and Rosenbaum, 2001; Watanabe et al., 2004). Nevertheless, individual MTs of the axonemal bundle could shorten and reelongate without affecting the length of the entire flagellum. To address the question of how EB1 exchange and tubulin incorporation are related, we expressed EB1-NG in a strain co-expressing mCherry-α-tubulin incorporation are related, we expressed EB1-NG in a strain co-expressing mCherry-α-tubulin. Scale bar: 3 μm. (D) Bar graph showing the mean fluorescence intensity of EB1-NG at the tips of long (n = 12) and short (n = 12) flagella of long-short cell. Error bars indicate SD. Significance: p ≤ 0.01.

EB1-NG dwells for elongated periods of time at the flagellar tip

To characterize the diffusional behavior by which EB1 accumulates at the flagellar tip in greater detail, we used increased laser intensities, which bleached most EB1-NG particles entering the flagella within a few seconds; this prevented the accumulation of unbleached protein, enabling us to observe individual EB1-NG particles (Figure 6A; Supplemental Movie S4). The vast majority (>97%; n = 93) of the EB1-NG particles in the ciliary shaft bleached in one step, indicating the presence of a single EB1-NG (Figure 6A). In the flagellar shaft, most EB1-NG particles displayed a random back-and-forth motion with a one-dimensional (1D) diffusion coefficient of 1.06 μm²s⁻¹ (n = 41; Figure 6B; Supplemental Movie S4). A subset (~5%) of EB1-NG particles moved in an apparent preferred direction along the flagella, with some particles taking multiple subsequent steps in one direction (white arrows in Figure 6, Supplemental Figure S5A, and Supplemental Movies S5 and S6). Such particles were observed moving toward the flagellar tip or base; also, the displacement of the particles between frames was variable, and the runs were interrupted by one or more steps in the opposite direction. Thus these particles show characteristics typical for diffusion; however, considering the low probability of such directional runs by diffusion, we cannot exclude additional mechanisms promoting a directional movement of proteins along flagella. The latter is suggested by the parabolic distribution of the mean-square displacement over time for such particles (Supplemental Figure S5B).

Near the flagellar tip, EB1-NG motility was markedly reduced and interspersed with stationary periods (Figure 6A; Supplemental Movies S5 and S6). We averaged EB1-NG mobility at the tip and determined a diffusion coefficient of D = 0.063 μm²s⁻¹ ± 0.033 μm²s⁻¹ based on 14 trajectories of EB1-NG particles moving in the distal 1-μm segment of the flagellum (Figure 6B). EB1-NG particles became transiently trapped in the tip region, and an average resident time of 2.5 s (SD 1.6 s, n = 51) was determined for those that could be tracked from entry to exit (Figure 6A, a, d, and e). Because many particles were bleached while being trapped in the tip region (Supplemental Figure S5), the true average dwell time of EB1-NG at the tip is likely to be longer.

Simulating EB1 accumulation at the flagellar tip

We wondered whether the observed differences in EB1-NG mobility are sufficient to explain its accumulation at the flagellar tip. We used a one-dimensional model, assuming the flagellum as a line of 12 μm in length, which is divided in a 11-μm-long proximal segment in which particles diffuse with a coefficient of 1.06 μm²s⁻¹ and a 1-μm-long distal tip segment with a diffusion coefficient of 0.063 μm²s⁻¹ (Figure 7A). At the beginning of the simulation, 100 particles were introduced into the proximal end of the model flagellum (Supplemental Movie S7). These parameters caused ~55% of the particles to Figure S4, and Supplemental Movie S3), while EB1-NG returned to the tip at standard rates. The data reveal that only small amounts of tubulin are incorporated into the axoneme of steady-state flagella. Formally, the experiment does not exclude the possibility that bleached mCherry-α-tubulin is released from the tip and reincorporated after GDP-to-GTP exchange. However, considering the high rate of entry and diffusional mobility of FP-tagged tubulin in flagella (Craft et al., 2015), one would expect a considerable incorporation of the tagged tubulin into flagellar tips. We interpret the data to the effect that the presence of EB1 at the tips of steady-state flagella does not depend on the de novo addition of tubulin to the axoneme.
accumulate in the tip segment (Figure 7B), a value similar to the ~62% (SD 6.6%, n = 14) determined for EB1-NG based on the fluorescence intensity; the somewhat higher value might reflect the omission in our measurements of the proximal portions of the flagella, which were out of the range of the TIRF excitation. In the simulation, particles remained an average of 5 s (SD 6.95) in the tip segment compared with 2.5 s in EB1-NG bleaching experiments; the latter were performed at high laser intensities, limiting the time span during which particles could be observed and thereby eliminating longer dwell times from our data.

We wondered to what extent EB1 accumulated at the tip simply because the direction into which particles can travel is restricted. In simulations assuming the same diffusion coefficient (1.06 μm²/s⁻¹) along the entire length of the flagellum, the concentration of particles in the distal segment was only very slightly elevated compared with the flagellar shaft (Figure 7, C and D). In summary, the simple model essentially recapitulates the experimental results on EB1-NG. We conclude that the distinct motilities of EB1 in the flagellar shaft and tip segment are sufficient to explain its accumulation at the flagellar tip without the need of motor-driven transport.

**DISCUSSION**

**EB1 transiently binds to the flagellar tip with long dwell times**

In vivo imaging was used to analyze the behavior of the MT plus-end tracking protein EB1 in flagella of *C. reinhardtii*. Similar to observations on singlet MTs, EB1-NG transiently attaches to the flagellar tip, most likely binding to the distal portions of axonemal MTs. However, we observed several features distinguishing EB1-NG behavior at the flagellar tip from that of EB1 at the end of growing cytoplasmic MTs. Compared with the latter, the tips of steady-state flagella accumulate only small amounts of EB1, which were only slightly elevated in growing flagella, indicative of a comparatively limited number of axonemal binding sites for EB1. *Chlamydomonas* flagella elongate with a maximum rate of ~400 nm/min compared with rates of 8 μm/min and more determined for cytoplasmic singlet MTs (Srayko et al., 2005; Bhogaraju et al., 2014). Assuming a similar GTPase activity of tubulin in cytoplasmic and flagellar MTs, the slow growth rate of the latter will minimize the size of any GTP/GDP+Pi tubulin zone, putatively restricting EB1 binding. EB1 has been used as an indicator for the presence of GTP-tubulin, and the presence of EB1 at the tips of steady-state flagella raises the question of whether axonemal MTs permanently maintain a GTP cap (Seetapun et al., 2012). In *C. reinhardtii*, tubulin turnover at the axonemal tip is a rather slow process: It takes dozens of minutes before hemaggulutinin-tagged tubulin introduced into unlabeled flagella using sexual cell fusion becomes incorporated to detectable levels (Marshall and Rosenbaum, 2001; Lechtreck et al., 2013b). Similarly, we showed that little or no recovery of fluorescence occurs after photobleaching of mCherry-α-tubulin in steady-state flagella. Also, one would expect that the EB1 signal is lost or diminished during flagellar shortening, when the axoneme depolymerizes and any GTP-tubulin maintained at the tip should be lost. EB1, however, remains attached to the tips of shortening flagella and displays unaltered exchange rates in FRAP experiments (Pedersen et al., 2003; this study). EB1 binding to the tips of nongrowing flagella is therefore unlikely to indicate the presence of GTP-tubulin. To solve this conundrum, we propose that the tip of the axonemal MT has a lattice conformation allowing for EB1 binding independent of the GTP status of tubulin. Indeed, EB1 binding is not strictly linked to the GTP state of tubulin: The EB1 comets observed on growing singlet MTs exceed the presumed GTP/GDP+Pi cap in length, suggesting a delay...
between GTP hydrolysis and the conformational changes in the lattice that will abolish EB1 binding (Maurer et al., 2014). The tips of axonemal MTs are crowned by cap structures that surround the MTs and insert plugs into the MT lumen (Dentler and Rosenbaum, 1977). These cap structures could generate a microtubular lattice to which EB1 can bind. Notably, these cap structures are maintained during flagellar shortening and therefore could preserve the EB1-binding sites as they track depolymerizing axonemal MTs. Compared with the transient binding sites at the end of rapidly growing singlet MTs, which are short-lived due to the chemical instability of GTP-tubulin in the lattice, the stable binding sites proposed here for axonemal MTs should result in different EB1 exchange characteristics. Indeed, EB1-NG was trapped for seconds in the distal flagella segment compared with mean dwell times of just 0.05 s determined in vitro for EB1 at the tips of growing singlet MTs or along GTP-γS MTs (Bieling et al., 2008; Chen et al., 2014). The possibility that EB1 binds to nontubulin tip proteins cannot be excluded, but the observation that growing flagella bind more EB1 links its binding to the conformation of the axonemal MT lattice.

In C. elegans sensory cilia, tubulin exchange at the microtubule tips of the middle and distal segments is clearly detectable after a few minutes (Hao et al., 2011). EB1-GFP localizes to these ciliary microtubule ends but does not recover to detectable levels after photobleaching, and movement of EB1-GFP in cilia was not observed. EB1 in C. elegans cilia could firmly reside at the sites of de novo tubulin incorporation. This contrasts our observations in C. reinhardtii, which show rapid EB1-GFP recovery but only little incorporation of tubulin in steady-state cilia. It is unknown whether the elaborate tip structures observed in many motile cilia are present in C. elegans sensory cilia or other primary cilia. Additional studies are required to determine whether (motile and nonmotile) cilia have principally different tip structures, tubulin exchange rates, and EB1 dynamics.

**EB1 moves into and inside flagella by diffusion**

In imaging experiments, EB1-NG did not comigrate with the IFT-B complex protein IFT20, and FRAP of EB1-NG at the flagellar tip was not affected when IFT was switched off using conditional mutants. IFT-like transport of EB1-FP was observed only once, likely representing an unusual event that might have been caused, for example, by clumping with a genuine IFT cargo. We conclude that EB1 enters and moves inside C. reinhardtii flagella independently of IFT. The transition zone at the flagellar base is thought to function as a diffusion barrier for large cytoplasmic proteins (Kee et al., 2012; Breslow et al., 2013). Despite the predicted molecular weight of ~95–120 kDa for EB1-FP (hetero-)dimers, the Stokes radii of their globular entities are below the estimated size-exclusion limit, suggesting that EB1 can freely diffuse from the cell body into the flagellum. Previously, we showed that GFP-tagged tubulin dimers (~130 kDa) diffuse apparently freely into C. reinhardtii flagella (Craft et al., 2015). Tubulin, however, is also a cargo of IFT, and vast amounts of tubulin are transported via IFT during flagellar growth. The elongation of the axonemal MTs will remove soluble tubulin from the flagellar matrix, generating a diffusional current that results in the net entry of tubulin from the cell body into the flagellum. However, diffusion alone is apparently insufficient to supply enough tubulin for flagellar growth. We propose that the different modes of transport observed for tubulin and EB1 reflect differences in the subcellular distribution of these proteins: estimates suggest that the concentration of soluble EB1 in the flagellum is similar to that in the cell body (see Materials and Methods). Binding sites at the flagellar tips and the end of growing cytoplasmic MTs will then locally accumulate EB1. In contrast, the tubulin concentration in the flagellar matrix, in particular the matrix of growing flagella, is likely to exceed that in the cell body cytoplasm (Craft et al., 2015). This suggests that IFT functions in concentrating soluble tubulin inside the flagellar matrix above cell body levels; a high tubulin concentration could be necessary to promote an efficient elongation of the axoneme.

Flagellar assembly and maintenance requires a mix of diffusion and motor-driven protein transport. It is of interest to determine which flagellar proteins move by diffusion, by IFT, or a combination of both, and whether rules exist allowing one to predict which mode of transport will be used by a particular protein (Figure 8). One prediction would be that proteins with elevated concentrations in the flagellar matrix versus the cell body cytoplasm will be transported...
A diffusion-to-capture mechanism is sufficient to accumulate proteins in flagella

Simulations in model flagella consisting of two regions with distinct mobile behavior essentially recapitulated our in vivo observations that a diffusion-to-capture mechanism is sufficient to explain the accumulation of EB1 at the flagellar tip.

In the case of EB1, the binding sites in the tip region are permanent, but it is worthwhile to consider situations in which the interaction between a diffusing protein and its flagellar binding sites are regulated. On the activation of such binding sites, the protein will rapidly accumulate by diffusion and, when binding is abolished, the released protein will exit the flagellum until the equilibrium concentration is reestablished. Such a mechanism appears to drive the light-regulated import and export of membrane-associated signaling proteins such as arrestin from the cilia-like outer segment of rods (Calvert et al., 2006). Regulated binding to the ciliary tip is also characteristic for the hedgehog signaling protein Gli; kinesin Kif7 is dispensable for Gli transport but maintains a Gli-binding compartment at the ciliary tip (He et al., 2014). Diffusion and regulated capture could explain the delivery of building blocks into growing cilia and the transient accumulation of signaling proteins in cilia without evoking IFT involvement.

**MATERIALS AND METHODS**

**Strains and culture conditions**

*C. reinhardtii* was maintained in batch cultures in a modified M (minimal media) at 21°C with a light/dark cycle of 14:10 h. For flagellar isolation and in vivo TIRF microscopy experiments, cultures were aerated and supplemented with 0.5% CO2.

The following strains were used in this study: wild-type (CC-620 and CC-621), *fla10-1* (CC-1919), *fla11-1* (CC-1920), *ifi20-1* IFT20-mCherry (Lechtreck et al., 2009), and mCherry-α-tubulin in CC-620 (Craft et al., 2015).

**Transgenetic strain generation**

For expression of fluorescent protein–tagged EB1, a 6-kb genomic DNA fragment, encompassing EB1 including 1.2 and 1.7 kb of the 5’ and 3’ flanking sequence, was amplified by PCR using a *Chlamydomonas* BAC clone as a template and primers (gcacagctgtctgaattgctgctggag) and (gtgctagcagctggtctcgaagcc), each containing an XbaI site. The PCR fragment was digested with XbaI and ligated into the complementary SpeI site in the pGEM-T Easy vector also containing a paromomycin (PMR)-resistant cassette to create the plasmid pCrEB1 (Zhu et al., 2013a). For tagging of the C-terminus of CrEB1, a 2.8-kb 3’ CrEB1 genomic DNA was amplified from the BAC clone using the primer pair (gcaagccccacgccgcaagccccagctg) and (gcagcagctgcagcagcagctg), and TA cloned into pGEM-T vector (Promega). A QuikChange site-directed mutagenesis kit (Stratagene, San Diego, CA) was used to convert the sequence before the stop codon into a plasmid pCrEB1 (Zhu et al., 2013a).
GFP-encoding fragment derived by PCR from pKL3-GFP was inserted (Lechtreck et al., 2009). The tagged DNA was reamplified to add a KpnI site through an antisense primer (taggtacccgagcctgagccaccagg). After digestion with KpnI, the tagged PCR fragment was inserted in pCrEB1, replacing the corresponding untagged fragment in pCrEB1 to create the plasmid pCrEB-GFP. For generation of the EB1-NG derivate, NG DNA was custom synthesized with I-digested NG plasmid using the primer pair (ctcgagatggtgcttcaaggg and ctcagctggtcagctgctc) with added XhoI sites (Craft et al., 2015). The XhoI-digested NG fragment replaced the GFP fragment in the pCrEB-GFP to create pCrEB-NG. An aliquot of these genomic constructs was transformed into Chlamydomonas cells using the glass bead method (Kindle, 1990), and positive transformants were selected on TAP plates containing 10 μg/ml PMM. The PMM-resistant clones were screened for fluorescence using a Nikon Eclipse wide-field microscope and a CoolSNAP-ES CCD camera. The fla10-1 EB1-GFP and fla11 EB1-GFP were generated and selected similarly. The IFT20-mCherry EB1-NG strain was generated by transforming IFT20-mCherry cells (Lechtreck et al., 2009) with the EB1-NG plasmid via electroporation. Positive transformants were selected on TAP media plates containing 10 μg/ml PMM, and resistant clones were screened via TIRF microscopy. The mCherry-α-tubulin EB1-NG coexpressing strain was generated by transforming EB1-mNeon cells with pBR25-mCherry-α-tubulin. The plasmid was constructed by PCR amplification of the mCherry gene from the pKL3-IFT20-mCherry construct described by Lechtreck et al. (2009). The amplified mCherry gene was digested with Xhol and BamHI and inserted into the pBR25-sfGFP-α-tubulin expression vector, replacing the sfGFP fragment (Rasala et al., 2013; Craft et al., 2015). Positive transformants were selected on TAP plates containing 10 μg/ml zeocin in constant light and identified by TIRF microscopy.

Flagellar isolation and Western blotting
For preparation of whole-cell samples, the cell pellet from a 5 ml late-log phase TAP liquid culture was resuspended with 50 μl 10 mM HEPES buffer, followed by the addition of 100 μl 5X SDS–PAGE sample buffer and boiling for 5 min. After 2 μl 1.7 mg/ml phenylmethylsulfonyl fluoride was added, the insoluble remnants were removed by centrifugation, and the supernatant was processed for SDS–PAGE and Western blotting. Flagellar samples were prepared as previously described (Yang et al., 2008). The following antibodies were used for analysis: rabbit polyclonal anti-EB1 (1:5000; Pedersen et al., 2003), mouse monoclonal anti-IC78 (1:5000; King and Witman, 1990), and mouse monoclonal anti-α-tubulin (1:5000; Sigma). Western blots were developed using anti-mouse and anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (Invitrogen), and chemiluminescence images were captured and documented using a UVP Autochemi Bioimaging System (Cambridge, UK).

Immunoprecipitation of EB1-GFP
Isolated flagella from the EB1-GFP–expressing strain and a control strain were resuspended in HMEK (30 mM HEPES, 5 mM MgSO4, 25 mM KCl, and 0.5 mM EGTA) supplemented with protease inhibitor cocktail (Sigma-Aldrich). Flagella were lysed by adding an equal volume of HMEK plus 200 mM NaCl and 0.5%NP-40 for 30 min on ice, and the axonemes were removed by centrifugation (20,000 × g, 4°C for 10 min). The membrane+matrix fraction was incubated with GFP-nAB agarose slurry (GFP-nAB, Allele Biotechnology) equilibrated with binding buffer (HMEK, 150 mM NaCl) followed by nutation for 1 h at 4°C. The slurry was washed three times with binding buffer (HMEK, 150 mM NaCl), this was followed by three washes with wash buffer (HMEK, 200 mM NaCl). The bound proteins were eluted with 1 M glycine (pH 2.5) and were analyzed by SDS–PAGE followed by Western blotting or silver stain (Bio-Rad Technologies).

Flagellar regeneration and long-short cell generation
For obtaining cells with regenerating flagella, cells grown in M medium were deflagellated by a pH shock, pelleted via centrifugation, and resuspended in fresh M media (Lefebvre, 1995). Cells were allowed to regrow flagella at room temperature under constant light with gentle agitation. To delay the regrowth of flagella, we...
placed cells on ice until needed. Long-short cells were generated by passing cells in M medium repeatedly (~4–6 times) through a 26 G × ½ in. needle attached to a 1-ml syringe. This method resulted in a small percentage of long-short cells that were identified by microscopy.

In vivo microscopy
A Nikon Eclipse Ti-U inverted microscope equipped with a 60×/1.49 numerical aperture (NA) TIRF objective and a through-the-objective TIRF illumination system with 75-mW, 561-nm and 40-mW, 488-nm diode lasers (Spectraphysics) was utilized for in vivo imaging experiments (Lechtreck, 2013). The excitation light was filtered with a Nikon GFP/mCherry TIRF filter cube, and the two-color emission light was separated by using a splitting device (Photometrics Dual-View2). Photobleaching of flagella was accomplished using two approaches. For bleaching the entire flagellum, the 488-nm laser-emission intensity was increased to ~10% for 5–30 s. For bleaching a specific area of the flagellum, a focused 488-nm laser beam passing through the specimen in epifluorescence mode was used for < 2 s. Increased laser intensities were used to image individual EB1-FP molecules. For in vivo imaging, 8–10 μl of cells was placed in a 24 × 60 mm no. 1.5 coverslip and allowed to settle for ~1–3 min. Then a 22 × 22 mm no. 1.5 coverslip containing an equal volume of 10 mM HEPES and 6.25 mM EGTA (pH 7.4) was placed on top of the large cover glass to form an observation chamber. Cells were imaged at room temperature (~24°C) or after incubation at 32°C, using an objective heater (Biotech). Images were recorded and documented at 1–31 frames/s using the iXon X3 DU897 EMCCD camera (Andor) and Elements software package (Nikon). ImageJ (National Institutes of Health) with the LOCI Bio-formats Importer (University of Wisconsin) and Multiple Kymogram (European Molecular Biology Laboratory) plug-ins was used to generate movies and kymograms and to retrieve single frames from the Element ND2 files. Kymograms, individual frames for time-lapse series, and movies were cropped and adjusted for brightness and contrast in ImageJ and Photoshop (Adobe). All figures were assembled using Illustrator (Adobe). Movies were cropped, adjusted for brightness and contrast, rotated, and converted to 8-bit format in ImageJ. The corrected movies were exported as AVI files, and QuickTimePro was used for scene selection.

FRAP and fluorescence intensity analysis
For determination of the fluorescence intensity, videos were opened in ImageJ, and the flagellar tip region or another region of interest (ROI) was selected using the Rectangle tool. The fluorescence intensity inside the selected region was determined using the Plot Z-axis tool, and the data were exported into Excel. The fluorescence intensity in the ROI was corrected for the background fluorescence using ROI of the same size. For FRAP analysis, videos were imported into ImageJ, an ROI was selected with the Rectangle tool, and the fluorescence of the ROI was determined from the Plot Z-axis tool. After background subtraction in Excel, the highest intensity value before the bleaching event was set to 100%, and the recovery of fluorescence (as percentage of the prebleached value) was calculated. In a subset of movies, the fluorescence lost during the experiment was calculated using the unbleached flagellar tip of the same cell as an internal control.

Estimation of the cellular distribution of EB1 and tubulin
Western blotting indicated a ratio of 70:1 for EB1 in the cell body versus flagella. The cell body has a volume of ~250 μm³ compared with 0.75 μm³ for a 12-μm-long flagellum. However, the volume of freely accessible cytoplasm and flagellar matrix is likely to be considerably lower. We used ~20% cytoplasm for the cell body, with its numerous cell organelles and vesicles that exclude tubulin, and 50% for the flagellum, in which a considerable volume is occupied by the axoneme. Then the cell body cytoplasm is ~66 times larger than that of the two flagella. This suggests that EB1 has a similar concentration in the cell body and the flagellum. The concentrations of tubulin have been estimated earlier. In brief, the two flagella contain ~20% of the cell’s total tubulin, corresponding to an ~12 times higher concentration in the flagellar compartment compared with the accessible cytoplasm (~20% of the total cell volume). In steady-state flagella, ~10% of the tubulin is soluble; the share of soluble tubulin in the C. reinhardtii cell body is unknown but has been estimated to be ~40% or more in other cells. Assuming that ~60% of the cell body tubulin is polymerized, the concentration of soluble tubulin in the flagellar matrix is twice that of the cell body cytoplasm during steady-state and approximately four times higher during flagellar growth.

Calculation of EB1-FP diffusion coefficient
To calculate the diffusion coefficient of EB1-NG in flagella, we utilized the same methods described for DRC4-GFP and GFP-α-tubulin (Wren et al., 2013; Craft et al., 2015). In short, 11 movies were selected that illustrated a high number of diffusing particles, and specific trajectories were identified from those movies using the ImageJ plug-in Mosaic Particle Tracker (Sbalzarini and Koumoutsakos, 2005). For each trajectory identified, the ratio of total distance traveled versus end-to-end distance was calculated; particles were excluded if this ratio was greater than 2.0, as these particles were likely not diffusing but rather undergoing a type of directed transport. The mean-square displacement versus time was calculated using the remaining 41 trajectories.

Similarly, EB1-NG was analyzed diffusing with a markedly reduced motility within the most distal 1-μm segment, noted as the flagellar tip. From 14 trajectories analyzed, a diffusion coefficient of 0.063 μm²s⁻¹ was determined.

To analyze those particles that displayed a directionally biased translocation along the cilia shaft, we determined the diffusion coefficient from 14 such trajectories. In this case, trajectories were excluded if the resulting ratio of total distance traveled to end-to-end distance was < 4.0; the excluded particles were likely undergoing typical diffusion instead of directed translocation.

Simulations
The simulations of one-dimensional diffusion of EB1 were written in Python. One hundred particles were initialized to a position at the base of the flagellum (x = 0). At each time step, the position of each particle was updated by selecting a random step, Δx, from a Gaussian distribution with SD, σ = √2DΔt, where D is the diffusion constant and Δt is the time step. For our simulations Δt = 0.1 s. D is position dependent:

\[
D(x) = \begin{cases} 
1.06 \mu m^2 s^{-2}, & 0 \leq x < 11 \mu m \\
0.06 \mu m^2 s^{-2}, & 11 \mu m \leq x \leq 12 \mu m 
\end{cases}
\]

At the base (x = 0) and at the tip (x = 12), reflecting boundary conditions were used. That is, if the new position x + Δx was greater than 12 μm or less than 0 μm, the new position was set to x = 12 or 0 μm, respectively. For each time step, the number of particles in the tip region (11 μm ≤ x ≤ 12 μm) was counted. Each time a particle
entered the tip, a counter was started to keep track of how long it spent at the tip. When a particle left the tip, the dwell time was added to a list, and the counter was reset to zero. The list was then used to generate the histogram of dwell times. Simulations using a $D(x)$ of 1.06 $\mu$m$^2$ for the entire flagellum and simulations in which the low-mobility region is moved from the tip down into the flagellar shaft were generated similarly.

The movies were created by generating a point-spread function (PSF) at the position of each particle at each time step. In each frame, each particle was assumed to emit 500 photons corrupted by Poisson noise. For generating the image of a particle, an ideal pupil function, $P(\mu, \nu)$, was generated with radius $NA/\lambda$, where the NA was chosen to be 1.2. $(\mu, \nu)$ is the position in the pupil plane and has units of 1/length. The ideal pupil is defined as

$$P(\mu, \nu) = \begin{cases} 1, & \sqrt{\mu^2 + \nu^2} < NA/\lambda \\ 0, & \text{otherwise} \end{cases}$$

The ideal pupil function was then multiplied by $\exp(2\pi i (\mu x + \nu y))$, where $(x, y)$ is the position of the particle. The image of the particle is then

$$\text{PSF}(x, y) = |F[P(\mu, \nu) \exp(2\pi i (\mu x + \nu y))]|$$

where $F$ denotes the Fourier transform. In this way, it is straightforward to generate subpixel particle movements. The PSFs generated by each particle are added to generate the image for each frame. A background of 20 photons is added to each pixel, and then Poisson noise is added to the image to generate the final image of each frame. The pixel size is 110 nm, and the number of pixels used to generate each image is $128 \times 128$.

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