Single particle imaging reveals IFT-independent transport and accumulation of EB1 in 

Chlamydomonas flagella

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Abstract

The microtubule (MT) +‐end tracking protein EB1 is present at the tip of cilia and flagella; 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 indicative for an exchange with unbleached EB1 entering the flagella from the cell body. EB1 moved independently 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.

**TOC summary**

The microtubule (MT) +−end tracking protein EB1 moves into flagella and accumulates at the tip independently of IFT. EB1 dwells for seconds at the tip indicative for the presence of stable EB1 binding sites. Simulations show that diffusion to capture is an alternative mechanism to accumulate proteins in cilia.

**Introduction**

Microtubules (MT) are polar assemblies of α- and β-tubulin. The MT +−end is more dynamic and various proteins bind to the +−end promoting MT elongation or shortening (Akhmanova and Steinmetz, 2010). End-binding protein 1 (EB1) is a widely distributed +−end tracking protein that binds directly to MTs *in vitro*. EB1 has been widely used to track the tip of growing MTs
and as an indicator for the presence of GTP/GDP+Pi tubulin near the +-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 also sensitive to the conformational state of tubulin in the MT lattice (Maurer et al., 2011).

The +-ends of the axonemal microtubules are at the distal tip of cilia 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 tip of motile 9+2 flagella, mammalian primary cilia, and sensory cilia in C. elegans (Pedersen et al., 2003; Hao et al., 2011; Schroder et al., 2011). These results reveal that, within both motile and primary cilia, EB1 accumulates on the tips of apparently static axonemal MTs contrasting its behavior on singlet MTs in the cytoplasm where EB1-binding is largely limited to the +-ends of growing MTs. In C. reinhardtii, EB1 remains at the tip 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 Tammana 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 tread-milling 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,
These observations raise the questions whether EB1 binding to the flagellar tip is similarly transient and depending on the addition of fresh tubulin as determined 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 tip 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 tip of non-growing flagella, the 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.

Here, we used *in vivo* imaging in *C. reinhardtii* to elucidate the dynamics of fluorescent protein (FP)-tagged EB1 in flagella. The FP-tag (GFP or mNeonGreen) 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 with its two flagella to cover glass allowing for the tracking of single fluorescence particles in flagella by TIRF microscopy (Lechtreck, 2013). IFT transport of EB1-FP was essentially absent and EB1-FP entered flagella by diffusion and transiently dwelled 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 green fluorescent protein (GFP) or the brighter mNeonGreen (NG) in wild-type cells (Fig 1 A; (Shaner *et al.*, 2013)). Western blotting of whole cells with anti-EB1 identified two bands of ~35kDa and ~60 kDa (Fig. 1 B). The former was also present in untransformed control strains and represents the endogenous untagged EB1 protein; the latter represents the EB1-GFP or EB1-NG fusion proteins. To determine the subcellular distribution of EB1, isolated flagella were probed with anti-EB1 (Fig. 1 B). Both endogenous and FP-tagged EB1 were present in flagella of transformants. To obtain a signal of similar intensity as the whole cell sample, ~70x more flagella sample (i.e. 140 flagella/cell body) had to be loaded suggesting that ≤2% of the total EB1 is present in flagella. EB1 is known to form dimers and we wondered whether the endogenous and transgenic EB1 interact with each other (Honnappa *et al.*, 2005). Using an anti-GFP nanobody, EB1-GFP was immunopurified from detergent extracts of isolated flagella (Fig. 1C). Endogenous EB1 remained attached to EB1-GFP after a medium stringency salt wash (200 mM NaCl) and silver staining of the eluate showed that EB1 and EB1-GFP are the predominate proteins in the eluate indicative for the presence of EB1/EB1-GFP complexes inside flagella, putatively in the form of EB1/EB1-GFP heterodimers (Fig. 1D).

TIRF microscopy of living cells attached via their two flagella to the cover glass showed FP-tagged EB1 concentrated at the tip of flagella (Fig. 1E) confirming previous observations on the endogenous EB1 based on antibody staining (Pedersen *et al.*, 2003; Sloboda and Howard,
Using steeper illumination angles allowed us to image EB1 in the cell body. EB1-NG was concentrated in the region of the basal bodies and present in a spotted distribution in more posterior regions of the cell (Fig. 1 F). In summary, the subcellular distribution of the endogenous EB1 is recapitulated by the FP-tagged protein indicating that the latter is a suitable reporter for the analysis of EB1 in vivo dynamics in C. reinhardtii.

**EB1-NG visualizes the dynamic properties of cytoplasmic MTs**

The microtubular cytoskeleton of C. reinhardtii consists of the axonemal MTs, the basal body MTs, and cytoplasmic or cortical MTs, which run from their origin near the basal bodies toward the posterior end of the cell (Doonan and Grief, 1987). EB1 tracks the + -ends of growing MTs in various cell types and thus can be used as a tool to visualize MT dynamics. EB1-NG comets were observed radiating from the basal body region and moving progressively to the posterior end of the cells revealing the hitherto unknown dynamics of the cortical MTs in C. reinhardtii (arrowheads in Fig. 2A; movie 1). Kymograms were used to determine the velocity of EB1-NG comets (Fig. 2B); the average velocity was 0.142 μm/s (±0.04 μm/s, n= 31; Fig. 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 cell end, 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 vanishing of the comets, 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 (Fig. 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 +-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. The 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 (Fig. S1) or only the flagellar tip (Fig. 3A); 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 approximately 3 - 7 minutes (Fig. 3B, C). Complete or near complete recovery was also observed after repeated bleaching of the flagellar tip (Fig. S1). In conclusion, EB1 at the flagellar tip is continuously exchanged with unbleached protein from the flagellum and the cell body.

Intraflagellar transport (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 (％ recovery of the prebleach signal intensity/minute) instead of total recovery time was used to compare EB1-FP recovery at different conditions (Fig. 3E, 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 temperature (32°C; Fig. 3D, F). Western blotting confirmed that the temperature shift was effective in abolishing IFT: At the restrictive temperature selected IFT particle proteins were quantitatively removed from *fla10-1* flagella; the levels of endogenous EB1 remained constant (Fig. 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 tip 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 tip and recovered normally after photobleaching in *fla11-1* cells maintained at 22°C and 32°C (Fig. S2A 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 levels were strongly reduced (Fig. 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 tip (not shown), 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 (Fig. 3G). EB1-NG also moved independently of the bona-fide IFT cargo mCherry-α-tubulin in steady-state and growing flagella (Fig. S3). During 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 RPE 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 tip 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 non-growing MTs. However, EB1 is present at the tip of steady-state, growing, and even shrinking flagella (Pedersen et al., 2003) raising the question whether the mechanisms of EB1 binding to axonemal and cytoplasmic MTs are different. We compared the intensity of the EB1-NG signal at the tip of steady-state flagella to that of the EB1-NG comets in the cells body. The latter is likely to represent the tip of a single MT while the former contains 9 A- and 9 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 A.U. vs. 257 A.U. for comets and flagellar tips; STD 89.9, n=4 and STD 74.0, n=10, respectively). To test if EB1-NG might be predominately attached to the two CP MTs, we expressed EB1-NG in the CP-deficient mutant *pf18*; the amounts and dynamics of EB1-NG at the tip of *pf18* flagella were essentially unaltered (not shown). In conclusion, only small amounts of EB1 are present at the flagellar tip suggesting that the +-ends of axonemal MTs in steady-state flagella attract considerably less EB1 than the tips of growing singlet MTs in the cell body.

To determine 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. To allow for a direct comparison of signal strengths, cells with regenerating and steady-state flagella
were mixed prior to imaging (Fig. 4A). The EB1-NG signal at the tip of regenerating flagella was on average 2.5x brighter than that of steady-state flagella (Fig. 4B) and often extended into the flagellar shaft. FRAP analysis of growing and steady-state flagella showed similar rates of EB1-NG exchange (Fig. S2 D). 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 tip of the longer, putatively retracting flagella and the EB1-NG signals at the tip of growing flagella of such long-short cells were increased ~2-fold in strength, often extending into the flagellar shaft (Fig. 4C, D). These single cell experiments show that the tips of flagella with elongating axonemes have an increased capacity to attract EB1-FP.

Limited turnover of axonemal tubulin in steady-state flagella appears unrelated to EB1 binding

The increased presence of EB1-NG at the tip of elongating flagella suggests a causal link between tubulin polymerization and EB1-NG binding to axonemal MTs raising the question whether EB1 accumulation at the tip 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 re-elongate without affecting the length of the entire flagellum. To address the question how EB1 exchange and tubulin incorporation are related, EB1-NG was expressed in a strain co-expressing mCherry-α-tubulin to about ~10% of the total α-tubulin (Fig. 5, S3, S4). Recovery of the fluorescent signals representative for EB1 and tubulin was analyzed after photobleaching of the distal portion of the flagella. To ensure
that an incorporation of mCherry-α-tubulin can be detected while continuously imaging cells by TIRF, we first analyzed growing flagella (Fig. S3). We observed dense IFT trafficking of mCherry-α-tubulin and incorporation into the elongating axoneme; the EB1 signal at the flagellar tip recovered similarly fast. In bleached steady-state flagella, however, only very little or no incorporation of mCherry-α-tubulin was observed even after prolonged observation (up to 20 minutes; Figs. 5A-C, S4), while EB1-NG returned to the tip with 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 re-incorporated 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 tip of steady-state flagella does not depend on the de novo addition of tubulin to the axoneme.

**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 (Fig. 6A). The fast majority (>97%; n = 93) of the EB1-NG particles in the ciliary shaft bleached in one step indicative for the presence of a single EB1-mNeonGreen (Fig. 6A). In the flagellar shaft, most EB1-NG particles displayed a random back-and-forth motion with a 1D-diffusion coefficient of 1.06 µm²s⁻¹ (n=41; Fig. 6B). A subset (~5%) of EB1-NG particles moved apparently with a preferred direction along the flagella with some
particles taking multiple subsequent steps in one direction (white arrows in Fig. 6 and Fig. S5A). 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 (Fig. 5SB).

Near the flagellar tip, EB1-NG motility was markedly reduced and interspersed with stationary periods (Fig. 6A). We averaged EB1-NG mobility at the tip and determined a diffusion coefficient of $D = 0.063 \, \mu\text{m}^2\text{s}^{-1} \pm 0.033 \, \mu\text{m}^2\text{s}^{-1}$ based on 14 trajectories of EB1-NG particles moving in the distal 1-µm segment of the flagellum (Fig. 6B). EB1-NG particles became transiently trapped in the tip region and an average resident time of 2.5 s (STD 1.6 s, n=51) was determined for those which could be tracked from entry to exit (Fig. 6A a, d, e). Since many particles were bleached while being trapped in the tip region (Fig. 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 if 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 $1.06 \, \mu\text{m}^2\text{s}^{-1}$ and a 1-µm long distal tip segment with a diffusion coefficient of $0.063 \, \mu\text{m}^2\text{s}^{-1}$ (Fig. 7A). At the beginning of the simulation 100 particles were
introduced into the distal end of the model flagellum. These parameters caused ~55% of the particles to accumulate in the tip segment (Fig. 7B), a value similar to the ~62% (STD 6.6%, n=14) determined for EB1-NG based on the fluorescence intensity; the somewhat higher value might reflect that in our measurements the proximal portions of the flagella were omitted because they were out of the range of the TIRF excitation. In the simulation, particles remained an average of 5 s (STD 6.95) in the tip segment compared to 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 which extend 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 to the flagellar shaft (Fig. 7C, 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 + -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 to the latter, the tips of steady-state flagella accumulates 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 to 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 flagella 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 tip of steady-state flagella raises the question 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 HA-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 tip of shortening flagella and displayed unaltered exchange rates in FRAP experiments (Pedersen *et al.*, 2003; this study). EB1 binding to the tip of non-growing flagella is therefore unlikely to indicate the presence of GTP-tubulin. To solve this conundrum, we propose that the tip of the axonemal MTs 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 circumference 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 to 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 to mean dwell times of just 0.05 s determined \textit{in vitro} for EB1 at the tip of growing singlet MTs or along GTP-γ-S MTs (Bieling \textit{et al}., 2008; Chen \textit{et al}., 2014). The possibility that EB1 binds to non-tubulin 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 \textit{C. elegans} sensory cilia, tubulin exchange at the microtubule tips of the middle and distal segment is clearly detectable after a few minutes (Hao \textit{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 \textit{C. elegans} cilia could firmly reside at the sites of \textit{de novo} tubulin incorporation. This contrasts our observations in \textit{C. reinhardtii} showing 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 \textit{C. elegans} sensory cilia or other primary cilia. Additional studies are required to determine whether (motile and non-motile) 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 does not co-migrate 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 which might have been caused, e.g., 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 diffusional barrier for large cytoplasmic proteins (Kee et al., 2012; Breslow et al., 2013). Despite its predicted molecular weight of ~95-120 kD for EB1-FP (hetero-)dimers, the Stokes radii of its 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 kD) 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 resulting 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 tip 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 (Fig. 8). One prediction would be that proteins with elevated concentrations in the flagellar matrix vs. the cell body cytoplasm will be transported by IFT, while proteins that are in a diffusional equilibrium between cell and flagellum will not use IFT; a prerequisite is that the proteins in question can efficiently enter and exit flagella by diffusion. Similarly, small cell body proteins, which might leak into flagella by diffusion, might depend on IFT to be removed from flagella. The membrane-associated protein phospholipase D, for example, is retained to >99% in the cell body under wild-type conditions, but accumulates progressively in flagella when IFT is abolished (Lechtreck et al., 2013a). In conclusion, IFT could function in moving proteins that pass freely through the transition zone against cellular concentration gradients in and out of flagella.

Diffusion-to-capture 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 with respect to the amount of the EB1-NG protein trapped in the low mobility region at the tip and the dwell time of individual particles in this region. In the simulated kinetic, however, 35% of the particles required just 38 seconds to enter the tip region, which is faster than the experimental data on EB1-NG. The simulation commences with an empty tip region whereas in the FRAP experiments bleached molecules have to exit the tips in
order for unbleached EB1-NG to replace them. The rate limiting step of FRAP is clearly the slow exchange of photobleached molecules at the tip with unbleached molecules rather than supply of unbleached EB1-NG by diffusion. For EB1, the local concentration in the low mobility region is ~18x higher than in the rest of the flagellum and cytoplasm. The simulations support 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 worth to consider situations in which the interaction between a diffusing protein and its flagellar binding sites are regulated. Upon 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 re-established. 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.

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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 hours. 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), *ift20-1* IFT20-mCherry (Lechtreck *et al.*, 2009), and mCherry-α-tubulin in CC-620 (Craft *et al.*, 2015).

Transgenetic strain generation

To express 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 (gcacacgtctagattcgcactgccgtgagc) and (gtctagaccaggcatcggaagtggcttcggagcc), 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 (PMM)-resistant cassette to create plasmid pCrEB1 (Zhu *et al.*, 2013). To tag the C-terminus of CrEB1, a 2.8-kB 3’ CrEB1 genomic DNA was amplified from the BAC clone using the primer pair (gcaagaccggtgcacatgacagcg) and (ccagagcgactgacccaggcatcg) and TA-cloned into pGEM-T vector (Promega). A QuikChange Site-Directed mutagenesis kit (Stratagene) was used to convert the sequence before the stop codon into a XhoI site into which a GFP-encoding fragment derived by PCR from pKL3-GFP was inserted (Lechtreck *et al.*, 2009). The tagged DNA was re-amplified to add a KpnI site through an antisense primer (taggtacccagagcgtaagcagcagc) and
tagged PCR fragment was inserted in pCrEB1 replacing the corresponding untagged fragment in pCrEB1 to create the plasmid pCrEB-GFP. To generate the EB1-mNeonGreen derivate, mNeonGreen DNA was custom synthesized based on the mNeonGreen protein sequence using the *Chlamydomonas* codon bias and amplified from the pBR25-CrNG plasmid using the primer pair (ctcgagatggtgtcaagg) and (ctcgagtgtgctcgtcc) 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 beads method (Kindle, 1990) and positive transformants were selected on TAP plates containing 10 µg/ml paromomycin (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 10µg/ml PMM and resistant clones were screened via TIRF microscopy. The mCherry-α-tubulin EB1-NG co-expressing 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 in (Lechtreck *et al.*, 2009). The amplified mCherry gene was digested with XhoI 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 10µg/ml zeocin in constant light and identified by TIRF microscopy.
Flagellar isolation and Western Blotting

To prepare the whole cell samples, the cell pellet from a 5-ml late log phase TAP liquid culture were resuspended with 50μl 10 mM Hepes buffer, followed by the addition of 100μl 5X SDS-PAGE sample buffer and boiling for 5 min. After adding 2μl 1.7 mg/ml PMSF, the insoluble remnants were removed by centrifugation and the supernatant was processed for SDS-PAGE and Western blotting. Flagella samples were prepared as described (Yang et al., 2008). The following antibodies were used for analysis: rabbit polyclonal anti-EB1 (1:5,000; (Pedersen et al., 2003)), mouse monoclonal anti-IC78 (1:5,000;(King and Witman, 1990) and mouse monoclonal anti-α-tubulin (1:5,000; Sigma). Western blots were developed using anti-mouse and anti-rabbit-IgG conjugated to horseradish peroxidase (Invitrogen) and chemiluminescence images were captured and documented using a UVP Autochmi Bioimaging System (Cambridge, UK).

Immunoprecipitation of EB1-GFP

Isolated flagella from the EB1-GFP expressing strain and a control strain were resuspended in HMEK (30mM HEPES, 5mM MgSO₄, 25mM KCL and 0.5mM EGTA) supplemented with protease inhibitor cocktail (Sigma-Aldrich). Flagella were lysed by adding an equal volume of HMEK plus 200mM NaCl and 0.5%NP-40 for 30 minutes on ice and the axonemes were removed by centrifugation (20,000 g, 4°C for 10 minutes). The membrane+matrix fraction was incubated with GFP-nAB agarose slurry (GFP-nAB, Allele Biotechnology) equilibrated with binding buffer (HMEK, 150mM NaCl) followed by nutation for 1 hour at 4°C. The slurry was washed three times with binding buffer (HMEK, 150 mM NaCl) followed by three washes with wash buffer (HMEK, 200mM NaCl). The bound proteins were eluated with 1M 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

To obtain cells with regenerating flagella, cells grown in M media 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, cells were placed on ice until needed. Long-short cells were generated by passing cells in M media repeatedly (~4-6 times) through a 26G x ½ needle attached to a 1mL syringe. This method resulted in a small percentage of long-short cells which were identified by microscopy.

In vivo microscopy

A Nikon Eclipse Ti-U inverted microscope equipped with a 60xNA 1.49 TIRF objective and a through-the-objective TIRF illumination system with 75mW 561nm and 40mW 488nm 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 utilizing a splitting device (Photometrics DualView2). Photobleaching of flagella was accomplished using two approaches: to bleach the entire flagellum, the 488nm laser emission intensity was increased to ~10% for 5-30 seconds. To bleach a specific area of the flagellum, a focused 488nm laser beam passing through the specimen in epifluorescence mode was used for less than 2 seconds. Increased laser intensities were used to image individual EB1-FP molecules. For in vivo imaging, 8-10µl of cells were
placed in on a 24x60mm No.1.5 cover slip and allowed to settle for ~1-3 minutes. Then, a
22x22mm No. 1.5 cover slip containing an equal volume of 10mM HEPES, 6.25mM 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 heating
(Bioptechs). Images were recorded and documented at 1-31 fps using the iXon X3 DU897
EMCCD camera (Andor) and Elements software packing (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 utilized 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 cropping 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 in ImageJ. The corrected movies were exported as AVI files and QuickTimePro was used for
scene selection.

FRAP and fluorescence intensity analysis

To determine the fluorescence intensity, videos were opened in ImageJ and the flagellar tip
region or other regions 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,
a ROI was selected utilizing 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
prior to the bleaching event was set to 100% and the recovery of fluorescence (as percentage of the pre-bleached 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 vs. flagella. The cell body has a volume of \( \sim 250 \, \mu m^3 \) compared to \( 0.75 \, \mu m^3 \) for a 12-\( \mu m \) long flagellum. However, the volume of freely accessible cytoplasm and flagellar matrix is likely to be considerably lower. We used \( \sim 20\% \) 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 \( \sim 66x \) 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 about 20\% of the cell’s total tubulin corresponding to a \( \sim 12x \) higher concentration in the flagellar compartment compared to the accessible cytoplasm (\( \sim 20\% \) of the total cell volume). In steady-state flagella, \( \sim 10\% \) of the tubulin are soluble, the share of soluble tubulin in the *C. reinhardtii* cell body is unknown but has been estimated to the \( \sim 40\% \) or more in other cells. Assuming that \( \sim 60\% \) of the cell body tubulin are polymerized, the concentration of soluble tubulin in the flagellar matrix is twice that of the cell body cytoplasm during steady-state and \( \sim 4x \) higher during flagellar growth.
Calculation of EB1-FP diffusion coefficient

In order 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 1μm segment, noted as the flagellar tip. From the 14 trajectories analyzed a diffusion coefficient of 0.063μm²/sec was determined. To analyze those particles that displayed a directionally biased translocation, the diffusion coefficient was determined from 14 selected trajectories. In this case, trajectories were excluded if the resulting ratio of total distance traveled to end-to-end distance was less than 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. 100 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, dx, from a Gaussian distribution with standard
deviation, \( \sigma = \sqrt{2D\Delta t} \) where \( D \) is the diffusion constant and \( \Delta t \) is the time step. For our simulations \( \Delta t = 0.1 \text{s} \). \( D \) is position dependent.

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

At the base (\( x=0 \)) and at the tip (\( x=12 \)), reflecting boundary conditions were used. That is, if the new position \( x+dx \) was greater than 12\( \mu \text{m} \) or less than 0\( \mu \text{m} \), the new position was set to \( x=12 \ \mu \text{m} \) or \( x=0 \ \mu \text{m} \) respectively. For each time step, the number of particles in the tip region (\( 11 \mu \text{m} \leq x \leq 12 \mu \text{m} \)) 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 tip dwell times. Simulations using a \( D(x) \) of 1.06 \( \mu \text{m/s}^2 \) for the entire flagellum and simulation 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. To generate the image of a particle, an ideal pupil function, \( P(\mu, \nu) \), was generated with radius \( \text{NA}/\lambda \) where the numerical aperture (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

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

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

\[
\text{PSF}(x, y) = |\mathcal{F}\{P(\mu, \nu) \exp(2\pi j(\mu x + \nu y))\}|^2
\]
\[ \mathcal{F} \text{ denotes the Fourier Transform.} \] In this way, it is straightforward to generate sub-pixel 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 110nm and the number of pixels used to generate each image is 128×128.

References


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 mNeonGreen (NG) were integrated into the genomic DNA encompassing the EB1 gene including its endogenous promoter (pro) and terminator (ter). 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 as loading controls, to IC78 and α-tubulin. The flagella samples were 70x more concentrated than the whole cell
sample (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 co-purifies 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. Bar, 3µm. (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. Bar, 3µm.
**Figure 2)** Fluorescent EB1 localizes to comets in the cell body

(A, 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. Bar, 2µm. (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. Bar, 1µm 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. As described in other species, some residual EB1 remains attached to the length of the MTs in the *C. reinhardtii* cell body. The MT labeled by arrowheads is initially capped by EB1-NG (T0) and then retreats with time (T19-T37 in seconds). Scale bar, 2µm. (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, 1µm and 10 s. See Movie 1.
Figure 3

A

B

C

D

E

F

G

EB1-mNeon/IT12-mCherry

38
Figure 3) EB1 at the flagellar tip is rapidly exchanged independently of/unaided by IFT

A, B) Individual frames (A) and corresponding kymogram (B) from a FRAP experiment demonstrating the exchange of EB1-NG at the tip of steady-state flagella. (A) Images taken before (pre) and at various time points (0-320 in 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 which was used for FRAP analysis. In the kymogram (B) the flagellar tip and base and the bleaching step are indicated. Bars = 1µm 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 pre-bleach strength in about ~ 3 minutes. 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, 2µm and 10 s. E, 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) 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 independently from each other. The base of the flagellum (base) and the distal tip (tip) are marked. Scale bars, 2µm and 10 s.
Figure 4) Growing flagella tips attract more EB1

(A) TIRF image of two EB1-NG cells, one with steady-state (arrows) and one with regenerating flagella (arrowheads). Scale bar, 3µm. (B) Mean fluorescence intensity of EB1-NG in the tip region of steady state (SS; n=15) and regenerating (REG; n=17) flagella. (243 AU, STD 113 AU; n=17 vs. 90 AU, STD 43 AU, n=15); Error bars indicate SD. Significance, p≤0.0001. (C) TIRF image showing EB1-NG in the flagella of a long-short cell, the long flagellum is marked by an arrow, the short one by an arrowhead. Bar = 3µm. (D) Bar graph showing the mean fluorescence intensity of EB1-NG at the tip of long (n=12) and short flagella (n=12) of long-short cell. Error bars indicate SD. Significance, p≤0.01.
Figure 5) Recovery of EB1-NG is not linked to tubulin exchange at flagellar tip

(A-C) Gallery of individual frames (A), corresponding kymograms (B), and signal quantification (C) of a two-color FRAP experiment. A) The distal flagellar region of a cell co-expressing EB1-NG and mCherry-α-tubulin was bleached using a laser spot which was moved along the flagellum (indicated by the dashed region) and FRAP was analyzed over several minutes (T0-T900 in s). Top row, EB1-NG; bottom, mCherry-α-tubulin; arrowheads, flagellar tip. Scale bar, 1μm. (B) Single channel kymograms corresponding the panel A. The bleaching step and the orientation of the flagella are indicated. Bars = 1μm 10 s. C) Quantification of the fluorescence intensity of EB1-NG (green) and mCherry-α-tubulin (red) corresponding to the photobleaching experiment depicted in A and B. The prebleach fluorescence intensity was set to 100 for both proteins; EB1-NG recovered rapidly and completely while only traces of mCherry-α-tubulin were recovered even after 15 minutes of observation. See Fig. S4 for a similar experiment.
Figure 6) Differential mobility of EB1-NG explains its accumulation at the flagellar tip.

A) Gallery of kymograms depicting diffusion of EB1-NG in flagella. Open arrows, particles with reduced mobility near the flagellar tip. Open arrowheads, bleaching events. White arrows, EB1-NG particles preferably moving in one direction along the flagellar shaft. White arrowhead: Reappearance of photobleached EB1-NG as it is infrequently observed for NG-tagged proteins. Note the reduced mobility of particles approaching the tip (compare c to d and
e), transiently stationary EB1-NG at the tip (a, b, d, e), and the differences in the time EB1-NG remains trapped at the tip. Scale bars (a-b), 1μm and 2 s; (c-e), 1μm and 1 s. B) Mean square displacement vs. time plots for EB1-NG particles moving in the flagellar shaft (open squares; n=41) or tip segment (filled squares; n=14).
Figure 7) Modeling EB1 distribution in flagella

The flagellum was modeled as a 12 µm-long line with a 1 µm long low mobility region (diffusion coefficient 0.06 µm² s⁻¹) at the tip and assuming a diffusion coefficient of 1.06 µm² s⁻¹ for the flagellar shaft of 11 µm. 100 particles were released at the base of the line at T0. A) Individual frames from a simulation. The line on the left indicates the position of the flagellar shaft and the low mobility region. See corresponding movie S7. B) Plot of the fraction of particles in the tip segment vs. time based on the simulation described in A. Green line: Expected share of particles in 1/12 of the flagellum assuming random distribution of the particles. C) Left: To explore to which extent the geometry of the tip will result in an accumulation of particles, we performed a similar simulation as in panel A but using the same particle diffusion coefficient along the entire flagellar length. Note the minimal accumulation of
particles at the flagellar ends in this maximum intensity projection over about 1000 frames.

Right: A similar maximum intensity projection but using the conditions described in A. D) Plot of the fraction of particles in the tip segment vs. time based on the single diffusion coefficient simulation described in C. Green line: as in B. E) Histogram showing the distribution of dwell times in the tip region based on the simulation described in A.
Figure 8) The role of diffusion and IFT in flagellar protein transport

A) Proteins with similar concentrations in the cell body cytoplasm and the ciliary matrix might not require IFT for transport. An example is EB1, which accumulates locally by being captured onto microtubule plus-ends.

B) Proteins in which the concentration in the ciliary matrix exceeds that in the cell body cytoplasm require IFT to be concentrated in the ciliary compartment. An example is tubulin, which enters cilia by diffusion and by IFT (Craft et al., 2015). IFT of tubulin and the tubulin concentration in the ciliary matrix are elevated during ciliary growth presumably to allow for an efficient elongation of the axoneme.

C) Many proteins are abundant in the cell body but efficiently excluded from flagella. If such proteins are able to enter cilia by diffusion, IFT might function as a scavenger exporting such proteins from cilia. An example is phospholipase D, which is retained to >98% in the cell body.
of *C. reinhardtii* and removed from cilia in an IFT- and BBSome-dependent manner (Lechtreck et al., 2013).

In summary, IFT functions in moving proteins against concentration gradients into and out of cilia. While this is likely to apply to small proteins, which are able to diffuse through the transition zone, larger proteins might depend on IFT to pass through the transition zone.