THE CONTRIBUTIONS OF DIFFUSION AND IFT IN CILIARY PROTEIN TRANSPORT

by

JAMES AARON HARRIS

(Under the Direction of Karl F. Lechtreck)

ABSTRACT

Cilia are cellular extensions composed from over a thousand distinct proteins. The composition of the cilium is regulated and many of the functions of the organelle depend upon a precise protein content. During assembly, a vast amount of proteins must be transported from the cell body to the site of assembly within the cilium. Small proteins enter cilia by diffusion and intraflagellar transport (IFT), the predominant protein transport system in cilia. This applies to tubulin, the main structural protein of cilia. How individual proteins utilize these two delivery modes as well as their respective contributions in ensuring the proper protein environment of the cilium remains unknown. To investigate the roles of diffusion and IFT in ciliary protein transport we utilized single molecule in vivo imaging of fluorescently tagged ciliary proteins. We showed that the microtubule end-binding protein, EB1, localizes to the tips of cilia and is continuously exchanged between the cell body and cilia in an IFT-independent manner. EB1 moves swiftly along the ciliary shaft but displays a markedly reduced mobility near the ciliary tip. These two distinct mobilities were sufficient to accumulate a protein at the ciliary tip in simulations. In contrast to EB1, tubulin enters cilia by diffusion and IFT. The roles of
diffusion and IFT in providing tubulin for axonemal elongation were determined using modified tubulin constructs and an altered IFT system. IFT transport was reduced by ~90% when the E-hook was removed from β-tubulin; transport was essentially abolished in conjunction with an altered IFT81 protein that cannot bind tubulin. Despite the extreme reduction in IFT, presence of E-hook deficient β-tubulin in the axoneme was marginally reduced indicating that the lion’s share of axonemal tubulin enters cilia via diffusion. We propose that the observed modes of transport utilized by proteins is a reflection of the subcellular distribution of said proteins. Proteins which are equally distributed between the cytoplasm and cilia utilize diffusion as a means to translocate between the two compartments. IFT, on the other hand, is employed to overcome concentration gradients to ensure high quantities of a protein near the ciliary tip to promote ciliary elongation.

INDEX WORDS: tubulin, diffusion, IFT
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1.1 CILIA IN HEALTH AND DISEASE

Cilia are evolutionarily conserved microtubule-based organelles that extend from the apical surface of nearly all cells in the vertebrate body. These organelles play a pivotal role in a multitude of essential processes including fluid propulsion in the brain, spinal cord and respiratory tract, cell signaling and communication, and embryonic development [1]. Additionally, cilia are utilized by protists, including the unicellular green algae *Chlamydomonas reinhardtii*, a model organism used in this study, for proper cell motility, division, and mating. In general, cilia can be divided into two main categories based on ultrastructure and function – motile and non-motile cilia. Nodal cilia, a combination of motile and non-motile cilia, are present in the early embryo and are vital in embryonic development. Next, I will discuss in more detail the different categories of cilia and the human diseases associated with defects in these organelles.

The majority of motile cilia possess a 9+2 axoneme with nine peripheral microtubular doublets and two central pair microtubules. Additionally, motile cilia possess a number of structures including radial spokes, outer and inner dynein arms, and nexin-dynein regulatory complexes (N-DRC) that are necessary for proper motility. These structures allow motile cilia to move in a planar whip-like fashion to propel whole organisms or fluid across a cell surface. In vertebrates, motile cilia can be found on the
epithelial surface of the respiratory tract, in the ventricles of the central nervous system, and the lining of Fallopian tubes [2]. In the brain ventricles and airways motile cilia help to sweep fluid or foreign particles through the structures. They are also responsible for the movement of oocytes in the female reproductive tract and for sperm motility in males [2]. Interestingly, the functional differences between motile and non-motile cilia are not as strictly demarcated as once thought. Certain motile cilia have been shown to also function in a sensory capacity – motile cilia in the mammalian respiratory epithelium use mechanosensation to sense changes in the viscosity of mucus and adjust ciliary mechanics to maintain a ciliary beat frequency that is sufficient to propel the more viscous mucus through the airways. [3-5].

Non-motile, or primary cilia, are present on most cells throughout the human body. As the name suggests, these cilia do not possess an intrinsic motility and are mostly defined by the canonical 9+0 axonemal pattern. However, while non-motile cilia do not beat they are critical organelles that sense a variety of external and internal cues ranging from light and odorants to mechanical stimuli [6, 7]. They play an important role in the regulation of cellular proliferation, differentiation, migration, polarity and morphology. Non-motile cilia are commonly referred to as the signaling hub of the cell due to the surface area-to-volume ratio and the sheer number of signaling proteins and ion channels distributed along the membrane; Hedgehog, Wnt, Platelet-derived growth factor (PDGF), and Hippo are some key pathways that are regulated through the cilium [8-10].

In the embryonic node, motile mono-cilia and non-motile primary cilia collaborate to establish the left/right asymmetry of the vertebrate body plan [2]. Unlike the previously discussed motile cilia, the motile cilia of the node possess a 9+0 axonemal
structure and move in a clockwise rotary pattern (this rotation is due to the presence of outer dynein arms in nodal motile cilia). Sidedness, the proper positioning of structures along the body axis, is a central theme throughout vertebrate development. The rotational movement of motile cilia generates a leftward flow of extracellular fluid in the node. This flow is sensed by the peripheral non-motile cilia and a response is created whereby an asymmetric cascade of gene expression across the node establishes the left-right asymmetry of organs in the vertebrate body [11]. The breaking of bilateral symmetry by nodal cilia is a critical step in the proper development of the heart and other processes of the embryo [12-15].

Due to their wide distribution throughout the human body ciliary defects result in a variety of disorders and diseases often with a multi-organ phenotype – these diseases are collectively termed ciliopathies. These disorders can result in blindness, developmental defects, hydrocephalus, infertility, cystic kidneys, polydactyly, obesity as well as heart defects. These defects are not solely due to the motile function of cilia but primarily include the role of cilia in sensing sensory cues from the extra- and intracellular environment. While the cilium is not a membrane-bound organelle the protein composition of the compartment is tightly regulated and only a specific subset of proteins are allowed entry. Mutations that result in an abnormal ciliary protein composition lead to human diseases such as nephronophthisis as well as Joubert and Meckel-Gruber syndrome [16, 17]. Similarly, defects in the assembly of cilia result in a host of disorders ranging from blindness and obesity to skeletal, renal and hepatic abnormalities [18-23]. Cilia are the oldest identified cellular organelle. However, it is only recently that their influence in human development and health has been discovered.
1.2 ULTRASTRUCTURE OF CILIA

The ultrastructure of cilia is characterized by the presence of three to four main components that are essential to the proper assembly and function of the organelle. Below, I will discuss in detail these regions and how they contribute to the organization and roles that are attributed to the cilium.

**Basal Bodies**

Cilia are nucleated from basal bodies, modified centrioles composed of nine short triplet microtubules (each containing tubules A, B, and C) arranged in a cylindrical fashion that anchor the cilia to the plasma membrane [24]. During mitotic division the basal bodies are used as centrioles for spindle formation. In non-cycling cells, however, the centriole matures into a basal body, migrates to the cortex of the cell and docks to the plasma membrane for ciliary formation [25]. After initiating ciliary assembly, basal bodies serve as the organizing center for IFT train assembly as many IFT proteins, motors and ciliary proteins accumulate in this region before entry into the cilium proper [26, 27]. The basal body region of the cilium ends with the termination of the C tubule and the A and B tubules continue throughout the transition zone and axoneme [28].

**Transition Zone**

Proximal to the axoneme is the transition zone. This region marks the transition from the triplet microtubules that comprise the basal bodies to the doublet microtubules characteristic of the axoneme. The transition zone is a complex region composed of many distinct structures assembled in a hierarchal order from three protein modules: the Joubert
(JBTS), Nephronophthisis (NPHP), and Meckel-Gruber (MKS) syndrome modules [17, 29-31]. The JBTS module is required for the assembly of the other NPHP and MKS modules. Many ciliopathies are caused by defects in proteins of these modules making this region a hot-spot for disease-causing mutations in humans [32].

Proteins of the transition zone including the transition fibers, Y-linkers, and the ciliary necklace together form the “ciliary gate” - a physical barrier between the cytoplasm and ciliary compartment [28, 33-35]. These structures help to link the microtubules of the axoneme to the membrane providing structural integrity to the transition zone. The transition zone is thought to maintain the protein composition of the cilia by influencing the entry and exit of proteins by physically restricting the size of proteins that can diffuse through the compartment (while estimates vary it is generally thought that proteins larger than 50kDa are not able to pass freely through the transition zone) [36-38]. Additionally, aspects of IFT may be regulated through the transition zone as IFT proteins have been shown to accumulate on the transition fibers [26]. How the large structure of IFT trains maneuver through the restricted area of the transition zone for entry into the cilia is currently unknown.

**Axoneme**

Distal to the transition zone is the axoneme which accounts for the majority of the total ciliary length. The axoneme is characterized by nine parallel doublet microtubules that, in motile cilia, are arranged radially around a pair of singlet microtubules known as the central pair [28]. The A tubule of each doublet is a complete microtubule composed of 13 protofilaments whereas, the B tubule consists of only 10 protofilaments and is
therefore, an incomplete microtubule. Multiple protein complexes such as the inner and outer dynein arms, radial spokes and dynein-regulatory complex (N-DRC) are associated with the A tubule of the doublets and this interaction is vital to the motility of the cilia [39]. In addition to serving as the scaffold for many ciliary proteins, the doublet microtubules are used as tracks for the motors of the IFT system [40, 41]. Near the ciliary tip, the B tubules of the doublet microtubules terminate leaving the A tubules and the central pair to form the distal segment. The length of the distal segment varies considerably among species and is thought to play a role in the sensory function of the organelle [42-44].

Ciliary doublet microtubules, much like microtubules found in the cytoplasm, are composed of heterodimers of α/β tubulin and are intrinsically polar – the plus end of axonemal microtubules are located at the ciliary tip. However, differences between these two populations of microtubules exist. Axonemal microtubules are tremendously stable displaying resistance to cold treatment and depolymerizing agents – a characteristic not exhibited by microtubules in the cytoplasm. Additionally, axonemal microtubules display only a very low level of tubulin turnover at the ciliary tip and axonemal tubulin is highly decorated with post-translational modifications (PTM’s)[45, 46]. The high level of PTM’s along with the dense association of many large structural components is thought to contribute to the stability of the axonemal microtubules [47].

**Ciliary Tip**

Transmission electron microscopy analysis in *Tetrahymena* and *Chlamydomonas* has resulted in detailed knowledge of the structure of the tip region. The ciliary tip is
composed of the A tubules and central pair microtubules of the distal segment that are capped by complex macromolecular structures of mostly unknown identity. The central cap sits atop the plus ends of the central pair microtubules and connects the structure to the ciliary membrane [48-51]. It is composed of a plug-like structure that is inserted into the lumen of the central microtubules on which sits two distal plates [50]. A spherical bead resides on the distal plate and is attached to the ciliary membrane [49]. Likewise, distal filaments cap the plus ends of the A tubules and are characterized by a plug that inserts into the lumen of the tubule to attach the A tubule to the ciliary membrane [48, 50]. It is thought that these capping structures aid in the integrity of the cilium and help the organelle withstand the forces associated with ciliary motility – it is worth mentioning that nonmotile cilia do not display the same elaborate capping structures seen in motile cilia [28].

While much is known about the organization of the ciliary tip and its related capping structures very little is known about what roles it might play in ciliary function. The ciliary tip is thought to play a part in a variety of ciliary processes such as ciliary assembly and length regulation, IFT turnaround and cargo unloading, and cell signaling [52]. Numerous proteins localize to the ciliary tip where they function in length regulation and cell signaling [8, 53-55]. Additional proteins are present at the tips of cilia. However, the functions of these proteins remain unknown. The microtubule plus-end binding protein, EB1, is present at the tips of cilia [56-58]. In the cell body, microtubules show dynamic instability cycling through stages of rapid growth and catastrophe. EB1 binds to the plus-ends of these microtubules and actively tracks them only when they grow [59]. However, in cilia EB1 localizes to the tip region
independently of the ciliary growth state prompting one to question if axonemal microtubules undergo constant turnover at the tip [57]? Additionally, the constant presence of EB1 at the ciliary tip, even when cilia are actively resorbing, raises the question: Is EB1 dynamic at the ciliary tip or could the protein be firmly attached to the tip and remain there independently of ciliary growth conditions? Many ciliary proteins use IFT to enter and translocate in the cilium. Does EB1 require IFT to localize to the ciliary tip? While the function of EB1 in cilia remains unknown, how the protein localizes to the ciliary tip and the dynamics of EB1 in cilia are resolved in this dissertation.

1.3 CILIARY ASSEMBLY

Cilia are complex organelles that are composed of more than a thousand distinct proteins [60, 61]. Early work on protozoan flagellates found that these organisms would shed flagella after a brief incubation in an acidic medium [62]. Interestingly, it was shown that cells could regenerate this organelle de novo from a pool of precursor proteins in the cell body. Studies on the kinetics of this regeneration showed that fully formed cilia can be assembled in 90 minutes following pH shock [62]. After a short lag phase, ciliary assembly occurs rapidly with a constantly deaccelerating rate until the full length is reached [62]. Much like the kinetics of assembly, the synthesis of many ciliary proteins is highly upregulated following deflagellation – tubulin, for example, accounts for approximately 15% of total protein synthesis following cilia loss [63-65]. As cilia approach full length, protein synthesis is greatly reduced and only very low levels of tubulin synthesis is present during ciliary maintenance [65]. Because cilia lack the
machinery for protein synthesis, the extensive amount of proteins that are synthesized during ciliary regeneration must be transported from their origin in the cytoplasm to the cilium proper. These characteristics necessitate that a vast amount of proteins must be translocated into and throughout the organelle in the brief time frame established for ciliary assembly.

Cilia are not preassembled in the cell body and propelled outwards. Instead they are built by the addition of axonemal and other subunits to the elongating structure that projects from the cell body. Early work from the Rosenbaum lab showed that assembly of the organelle occurs at the distal end [66]. To examine the site of ciliary assembly, an HA-tagged version of tubulin was expressed in *Chlamydomonas* cells and the unique mating reaction was used to introduce the tubulin into cells with both growing and non-growing cilia [66]. The resulting quadriflagellate had a shared cytoplasm and the HA-tagged tubulin originally in the non-growing cilia had access to the now actively growing cilia. Immunofluorescence analysis from the resulting quadriflagellates found that the incorporation of HA-tagged tubulin was restricted to the distal plus tip of cilia – the growth zone [45, 66]. These observations indicate the need for a ciliary protein translocation system that could efficiently deliver the needed precursors to the growing end. Furthermore, many ciliary proteins are concentrated several thousand-fold in cilia. Thus, protein delivery solely by diffusion would be futile [60, 61]. Intraflagellar transport (IFT) was discovered in the early 1990’s and much work since then has shown that this active transport system is vital to the assembly of cilia and delivers axonemal proteins to the ciliary tip. These results show why IFT is thought be the predominant pathway to move proteins into and throughout cilia. Information on the proteins that comprise the
IFT system and how it associates with ciliary precursors for transport will be discussed next.

1.4 INTRAFLAGELLAR TRANSPORT

“Kozminski, Wake up! You need to look at Bloodgood’s Balls.” This phrase was spoken to Keith Kozminski by his Ph.D. advisor Joel Rosenbaum on an early morning train ride to Boston [67]. Unbeknownst to them this conversation would spark the discovery of a novel protein transport pathway that has since been shown to be vital in the assembly of cilia and flagella and has uncovered a link between cilia and human disease.

This transport pathway was termed intraflagellar transport, or IFT. Many axonemal and ciliary proteins utilize this system for import into the cilium. In definition, IFT is the bidirectional motor-driven movement of multimegadalton protein arrays inside cilia. This transport occurs between the ciliary membrane and the outer microtubule doublets of the axoneme by approximately 100-700 nm-long complexes termed IFT trains [40, 41, 68, 69]. IFT trains are polymers comprised from a double row of IFT particles which are themselves composed of 22 evolutionarily conserved IFT proteins that are arranged into biochemically stable subcomplexes termed IFT-A, IFT-B1 and IFT-B2 with 6, 10, and 6 proteins included in each subcomplex [70-72]. These complexes serve as cargo adapters of ciliary proteins for the IFT transport system. IFT proteins contain several domains that have been implicated in protein-protein interactions such as tetratricopeptide repeats (TPR), WD-40 repeats and coiled-coil domains [73]. Two molecular motors, kinesin-II and IFT dynein 1b are part of the IFT train and are
used to propel the complexes along the microtubules [74-77]. Additionally, the BBSome, a complex of eight proteins is a substoichiometric component of IFT trains and contributes to the export of membrane associated proteins [78-82].

The process of IFT can be subdivided into two pathways: anterograde and retrograde IFT. Anterograde IFT proceeds from the base of the cilium to the distal tip and is dependent on an active heterotrimeric kinesin-II and the IFT-B complex. Anterograde IFT trains and their respective ciliary cargo (including the IFT-A complex and IFT dynein 1b) are assembled in the basal body region from a cellular pool of IFT subunits before entering into the cilium proper [26, 83]. Recent work has shown that the anterograde IFT train assembly process occurs in stages with IFT-A and IFT-B forming a subcomplex to which the motors and ciliary cargo, specifically tubulin, attach briefly before the train enters the cilium [27]. At the ciliary tip, cargo proteins disassociate from the anterograde train and move to their respective assembly sites along the axoneme [83-85]. Additionally, anterograde IFT particles disassociate from one another following kinesin inactivation and the complexes are remodeled into less structured retrograde trains [69, 86, 87]. Retrograde IFT trains (including IFT subcomplexes B1 and B2) progress back from the ciliary tip to the base with this movement dependent upon IFT dynein 1b and the IFT-A complex of proteins. Interestingly, in C. reinhardtii, kinesin-II is not associated to retrograde IFT trains and instead diffuses back to the cell body [87]. Proteins destined for export from the cilium associate with retrograde trains and are thereby transported out of the cilium proper. Of note, anterograde and retrograde trains transverse up and down the cilia using the axonemal outer microtubules as tracks. With a diameter of approximately 200 nm, most of which is occupied with the axonemal
substuctures such as the large outer dynein arms, how is it that these large IFT trains, along with ciliary cargo, move efficiently throughout the ciliary highway and avoid collisions with other trains? To circumvent such collisions, it was recently shown that anterograde trains move exclusively along B-microtubules whereas retrograde trains travel back on A-microtubules [41].

Cilia do not contain ribosomes and the assembly process of cilia occurs at the distal tip. These results suggest that for efficient assembly to occur axonemal and other ciliary proteins would need to interact with the IFT system for transport into the organelle. Tremendous work has been carried out to identify ciliary proteins that are transported via IFT; for a list of proteins shown to move with IFT-like velocities see [88]. For a subset of these proteins, a direct interaction with specific IFT partners have been identified [89, 90]. One such interaction occurs between proteins of the IFT-B1 complex and tubulin. Bhogaraju et al. showed that the calponin homology (CH) domain of IFT81 and a basic domain of IFT74 form a tubulin-binding module utilized for the transport of tubulin within cilia [90]. The calponin homology (CH) domain of IFT81 binds to the globular interface of a tubulin dimer (Kd = 16 µM) while the acidic N-terminal domain of IFT74 interacts with the negatively charged C-terminal of β-tubulin; the latter interaction increases the binding 18-fold. The in vivo contribution of the IFT81-74 tubulin-binding module revealed that single mutants in either IFT74 or IFT81 (deletion and mutation of residues concerning tubulin binding, respectively) resulted in near normal ciliary length. However, the assembly process occurred at a much slower rate [91]. The lag in ciliary assembly could be explained by the reduced rate of IFT-based transport of α-tubulin reported in these mutants. Furthermore, double mutants combining the tubulin binding
mutations of IFT74 and IFT81 resulted in severely short or cells with no cilia suggesting that the IFT81-74 tubulin module provides the bulk of ciliary tubulin for assembly [91]. The specific contribution of the IFT81-74 module to the transport of β-tubulin by IFT and the process of ciliary assembly will be analyzed in a later chapter.

While our knowledge of IFT and its respective cargoes is expanding, many questions still remain: Do all ciliary proteins utilize IFT for entry into the organelle? IFT transports many ciliary precursors into the cilium. However, work in this dissertation reveals that not all ciliary proteins are dependent on IFT for their entry and localization. For those proteins that do interact with the IFT particle, how is the load of those proteins on the IFT particle regulated? Previous work from our lab has shown that IFT particles during ciliary assembly are heavily occupied with ciliary precursors, while during ciliary maintenance the IFT-cargo load is significantly reduced [84, 85]. What mechanisms are responsible for assuring that the cargo load on IFT is sufficient for the growth needs of the cilium? Additionally, why do proteins such as tubulin use IFT when the protein also enters the cilium by diffusion? Wouldn’t it be a better use of resources to use those IFT particles for proteins that are solely dependent on IFT for their ciliary entry? What is the contribution of diffusion and IFT in providing proteins for ciliary assembly and maintenance? The ability to observe the movements of both IFT and ciliary proteins in vivo combined with the capability to manipulate the known IFT-cargo interactions stated above positions us in the unique situation to begin to answer some of these unresolved questions.
1.6 CHLAMYDOMONAS AS A MODEL SYSTEM FOR CILIARY RESEARCH

Chlamydomonas reinhardtii provides an excellent platform to examine the properties of protein transport in cilia as well as other aspects of ciliary and general cell biology through a combination of biochemistry, genetics and fluorescence microscopy analysis. Chlamydomonas is a unicellular green algae with an ovate cell body encased in a cell wall and two approximately 12 µm long flagella which extend from the anterior end of the cell. Cells contain a sequenced haploid genome of approximately 120Mb. Genes shown to play a role in ciliary assembly such as IFT88 and known ciliopathy genes like polycystin-2 (PKD2) are conserved between C. reinhardtii and humans [19, 22, 61]. This conservation suggests that the fundamental rules regarding the regulation of ciliary assembly and protein transport in C. reinhardtii will likewise apply to the cilia of mammalian organisms. The DNA can be easily isolated from cultured cells for use in molecular cloning, i.e. tagging of ciliary genes with fluorescent fluorophores for visualization. Additionally, genes can be inserted into C. reinhardtii through electroporation, as well as other techniques, allowing for the creation of insertional mutants or the rescue of known mutants with native genes.

A plethora of mutants have been identified over the years in both IFT and potential cargoes of the system that provide a model for testing how alterations in IFT proteins can affect protein trafficking and ciliary growth and composition. In one such mutant, a conditional mutant of the anterograde IFT motor (fla10-1), IFT can be switched off by incubating the cells at the nonpermissive temperature [92]. This mutant has been a valuable tool for determining whether a ciliary protein requires an active IFT system for import into the organelle as well as the role of IFT in ciliary assembly and maintenance.
Additionally, a large and comprehensive insertional mutant library spanning 1562 genes was recently created allowing researchers to examine how defects in a multitude of ciliary genes affect the assembly and maintenance of cilia [93].

One of the major advantages of *Chlamydomonas* that is not available in other ciliary model organisms (besides protists) is that cilia can be removed and isolated from the cell. Deflagellation can be achieved through chemical or mechanical measures and results in the active regrowth of the cilia. Following isolation, cilia can be fractionated into axoneme, matrix, and membrane proteins for extensive quantitative biochemical analysis of protein composition through SDS-PAGE, silver and coomassie blue stain, and Western blotting procedures. This feature allows one to study how the *de novo* assembly of cilia influences properties of protein import through IFT and diffusion and how this may differ from what occurs during ciliary maintenance. Additionally, how mutations in various ciliary proteins alter the overall composition and stability of cilia can be determined utilizing an unbiased analysis of isolated cilia.

Importantly, *C. reinhardtii* cells are exceptionally well suited for *in vivo* protein imaging via total internal reflective fluorescence (TIRF) microscopy due to the cells’ natural gliding motility and cell structure. TIRF microscopy allows for the visualization of low abundance fluorescently tagged proteins through an evanescent excitation field created by the reflection of the excitation laser off the glass coverslip. The natural adhesion properties of the cells’ flagella brings the cilia (diameter of 200 nm) into the evanescence field of TIRFM (ranges from 20-300 nm) allowing for the illumination of proteins specifically inside the cilia; this evanescence field and the diameter of *Chlamydomonas* cilia prevents autofluorescence from the cell body and results in single
molecule imaging with unprecedented quality [94]. Characteristics of individual IFT protein movements during transport, interaction of ciliary proteins with IFT particles, and the differences in mobility of a single protein at the ciliary tip versus the ciliary shaft were determined by TIRF microscopy.

Equally important as the technical advantages afforded by using *C. reinhardtii* is the ease at which the algae can be handled, grown and stored. Cells can be efficiently cultured and synchronized in a grow room with a light-dark cycle and supplemented with CO₂. For long-term storage the algae can be kept on simple agar plates. This easy-to-use organism offers researchers of all experience levels a system to examine key aspects of ciliary biology.

1.7 OVERVIEW OF DISSERTATION

The processes guiding the assembly and maintenance of cilia are not only important for human health and disease but are likely guiding principles throughout cellular biology. Gaining basic information on the modes of transport proteins use to gain access to the cilium will provide a more comprehensive model of how the complex structure of cilia is assembled and maintained. Additionally, the molecular interactions used between tubulin, the most abundant ciliary protein, and the IFT particle will likely offer basic rules concerning how ciliary proteins bind to and are transported via the IFT system.

In Chapter 2 of this dissertation, we will describe how the microtubule end-binding protein 1 (EB1), utilizes diffusion, not IFT, to enter and accumulate at the ends of ciliary microtubules. Using TIRF microscopy and fluorescently labeled EB1 proteins we
show that EB1 displays two distinct phases of mobility and that these two phases, termed diffusion and capture, are sufficient to accumulate the protein at the tip of the cilium. From this data we propose that proteins, like EB1, that are uniformly distributed between the cytoplasm and cilia, can accumulate locally through diffusion and capture whereas, IFT might be used to transport proteins against cellular concentration gradients either into or out of the cilia.

Contrary to EB1, tubulin moves inside the cilium by diffusion and IFT. Tubulin and EB1 both fall under the size restriction imposed by the ciliary gate allowing both proteins to freely diffuse between the cytoplasm and ciliary compartment. Why is IFT needed to transport tubulin into cilia if diffusion is available? We examine the contributions of diffusion and IFT in delivering tubulin for ciliary assembly in Chapter 3 of this dissertation. For this aim we utilized TIRF microscopy and fluorescently-labeled tubulin constructs in which the C-terminal E-hook of the protein had been altered. Through this strategy we show that deletion of the E-hook of β-tubulin severely reduces the rate of transport by IFT, but reduces its presence in the axoneme only slightly. Additionally, we show that the vast majority of tubulin enters the cilium by diffusion; much more than previously expected. We hypothesize that the geometry of the cilium paired with the constricted opening from the cell body reduces the efficiency of tubulin entry by diffusion and that IFT is then required to finely tune the intraciliary concentration to ensure efficient ciliary assembly.

In Chapter 4, we summarize the entirety of the work completed in this dissertation. Additionally, we discuss how IFT transport of tubulin could regulate the length of cilia and contemplate potential functions of IFT during ciliary maintenance.
Our data reveal that diffusion has a large role in the assembly process of cilia. Through our work we demonstrate that ciliary assembly is a combination of diffusion and active transport. It is now imperative to tease out the contributions of each process to ciliary assembly and determine what regulates the mode of transport a ciliary protein uses.
CHAPTER 2

SINGLE-PARTICLE IMAGING REVEALS INTRAFLEGELLAR TRANSPORT-INDEPENDENT TRANSPORT AND ACCUMULATION OF EB1 IN

CHLAMYDOMONAS FLAGELLA

2.1 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.

2.2 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 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 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 cilia, 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 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
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 with its two flagella to a cover glass,
allowing for the tracking of single fluorescent particles in flagella by total internal reflection fluorescence (TIRF) microscopy (Lechtreck, 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

2.3 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., 2013). Western blotting of whole cells with anti-EB1 identified two bands of ~35 and ~60 kDa (Figure 1B). 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. For determination of subcellular distribution of EB1, isolated flagella were probed with anti-EB1 (Figure 1B). Both endogenous and FP-tagged EB1 were present in flagella of transformants. To obtain a signal of similar intensity as the whole-cell sample, ~70 times 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, we immunopurified EB1-GFP from detergent extracts of isolated flagella (Figure 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 predominant proteins in the eluate indicative for the presence of EB1/EB1-GFP complexes inside flagella, putatively in the form of EB1/EB1-GFP heterodimers (Figure 1D).

TIRF microscopy of living cells attached via their two flagella to the cover glass showed FP-tagged EB1 concentrated at the tips of flagella (Figure 1E), confirming previous observations on the endogenous EB1 based on antibody staining (Pedersen et al., 2003; Sloboda and Howard, 2007). 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 spotty distribution in more posterior regions of the cell (Figure 1F). 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*.

### 2.4 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 that run from their origin near the basal bodies toward the posterior end of the cell (Doonan and Grief, 1987). EB1 tracks the plus-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 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.

### 2.5 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.

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 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.

2.6 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 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 steadystate 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.

2.7 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 coexpressing mCherry-α-tubulin to about ~10% of the total α-tubulin (Figure 5 and Supplemental Figures S3 and 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 could be detected while continuously imaging cells by TIRF, we first analyzed growing flagella
(Supplemental Figure S3). We observed dense IFT trafficking and incorporation of mCherry-α-tubulin into the elongating axoneme; the EB1 signal at the flagellar tip recovered with similar speed. 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 min; Figure 5, A–C, Supplemental 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.

2.8 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.
2.9 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 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 μm²s⁻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

2.10 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 hemagglutinin-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-γ-S 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 it’s 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.

2.11 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 diffusional 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 \((\sim 130\) 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 and 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 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 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 in and out of flagella through the transition zone and against cellular concentration gradients.

2.12 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 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 s to enter the tip region, which is faster than the experimental data on EB1-NG. The simulation commences with an empty tip region, whereas bleached molecules have to exit the tips in order for unbleached EB1-NG to replace them in the FRAP experiments. 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 \( \sim 18 \) times higher than in the rest of the flagellum and cytoplasm. The simulations support our \textit{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.
2.13 FIGURES

Figure 1

A

![Diagram showing a gene structure with indica](image)

B

![Western blot analysis](image)

C

![Immunofluorescence images](image)

D

![Immunofluorescence images](image)
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 as loading controls, to IC78 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. 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.
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. 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: 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.
Figure 3

A

B

C

D

E

F
FIGURE 3: EB1 at the flagellar tip is rapidly exchanged independent of/unaided 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.
Figure 4

A

B

C

D

fluorescence intensity (AU)

SS

n = 15

REG

n = 17

fluorescence intensity (AU)

Long

n = 12

Short

n = 12

****

**
FIGURE 4: Growing flagellar tips attract more EB1.

(A) TIRF image of two EB1-NG cells, one with steady-state (arrows) and one with regenerating (arrowheads) flagella. Scale bar: 3 μm. (B) Mean fluorescence intensity of EB1-NG in the tip region of steady-state (SS; n = 15) and regenerating (REG; n = 17) flagella. (243 a.u., SD 113 a.u.; n = 17 vs. 90 a.u., SD 43 a.u., 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. 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.
FIGURE 5: Recovery of EB1-NG is not linked to tubulin exchange at the 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 coexpressing EB1-NG and mCherry-α-tubulin was bleached using a laser spot that was moved along the flagellum (indicated by the dashed region), and FRAP was analyzed over several minutes (T0–T900 s). Top row, EB1-NG; bottom, mCherry-α-tubulin; arrowheads, flagellar tip. Scale bar: 1 μm. (B) Single-channel kymograms corresponding to A. The bleaching step and the orientation of the flagella are indicated. Scale bars: 1 μm and 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 min of observation. See Supplemental Figure S4 for a similar experiment.
Figure 6

A

B

Mean Square Displacement (μm)

Time (s)

shaft

tip
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 with d and e), transiently stationary EB1-NG at the tip (a, b, d, and e), and the differences in the time EB1-NG remains trapped at the tip. Scale bars: (a and b) 1 μm and 2 s; (c–e) 1 μm 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 μm²s⁻¹) at the tip and assuming a diffusion coefficient of 1.06 μm²s⁻¹ for the flagellar shaft of 11 μm. One hundred 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 Supplemental 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 what extent the geometry of the tip will result in an accumulation of particles, we performed a simulation similar to 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 ~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

A  
[cell body] = [cilium]

B  
[cell body] < [cilium]

C  
[cell body] > [cilium]

diffusion

IFT
**FIGURE 8: The role of diffusion and IFT in flagellar protein transport.**

The saturation of the orange background color indicates protein concentration, with dark colors specifying high concentrations. (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 in 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., 2013a). 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.
2.14 SUPPLEMENTARY FIGURES

Figure S1
Figure S1: The EB1-FP signal recovers repeatedly after photobleaching.

A) TIRF image of a flagellum from a cell expressing EB1-GFP. The white box outlines the region used for FRAP analysis. B) Kymogram from the flagellum shown in (A); the orientation and the bleaching steps are indicated. Scale bar, 2μm. C) Measurement of the fluorescence intensity (as a percentage of the maximum intensity prior to bleaching) following repeated photobleaching of the entire flagellum.
Figure S2

A

B

C

D

IFT57
IFT172
EB1
IC78

IFT57
IFT172
EB1
IC78

IFT57
IFT172
EB1
IC78

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Figure S2: EB1-GFP presence and exchange at the flagellar tip are not affected in *fla11-1*.

A) Kymograms depicting the presence and recovery of EB1-GFP in *fla11* cells at the permissive (22°C) or restrictive (32°C) temperature. The flagellar orientation and bleaching events are indicated. Scale bars, 1μm and 1 s. B) Mean recovery rates from *fla11-1* cells expressing EB1-GFP at the permissive (22°C) and restrictive (32°C) temperatures for IFT172 function. Error bars indicate the SD. C) Western blot analysis of flagellar preparations obtained at the permissive (26 °C) and restrictive (32 °C) temperature from wild type (a), and the conditional IFT mutants *fla10-1* (b) and *fla11-1* (c). Western blots were stained with the antibodies indicated. Note the presence of endogenous EB1 and reduction of IFT proteins including IFT172 in cilia harvested from cells maintained at the restrictive temperature. D) The rate of EB1-NG exchange at flagellar tip is not affected by the growth state of the flagellum. Mean recovery rates of EB1-NG at the tip of steady-state (SS) and regenerating (REG) flagella in wild-type cells. Error bars indicate SD. No statically significant difference in recovery rate was observed under the two growth conditions.
Figure S3: EB1-NG and mCherry-α-tubulin recover after FRAP of growing flagella.

Cell expressing EB1-NG and mCherry-α-tubulin during flagellar regeneration. Shown are TIRF images with corresponding single and merged channel kymograms before (pre), during photobleaching of the entire flagellum (bleach) and at various time points (T1-T8 in minutes) after photobleaching. Dashed white lines serve as marks to better visualize flagellar growth over the course of the analysis. Drawings illustrate the change in cell adherence to the coverslip between in the first two panels. Scale bar, 1μm and 1 s.
Figure S4

bleach

EB1-NG

merged

mCherry-α-tubulin

EB1-NG/mCherry-α-tubulin
Figure S4: Comparison of EB1-NG and mCherry-α-tubulin exchange at the flagellar tip

Single and merged channel kymograms illustrating a FRAP experiment using a cell expressing EB1-NG and mCherry-α-tubulin with steady-state flagella. Note that EB1-NG recovers rapidly following photobleaching of the distal portion of the flagellum while mCherry-α-tubulin fails to recover over the course of the recording. The flagellar orientation and bleaching event are indicated. Scales bars, 1μm and 2 s.
Figure S5

A

B

EB1 shaft, total end-to-end < 4

Average of 14 trajectories

Line, $D = 1.4 \mu m^2/s$
Figure S5: Unusual diffusional properties of some EB1-NG particles in flagella

A) Kymograms (left) and manual tracings (right) of EB1-NG particles in flagella. Fast moving particles are marked by arrows and the binominal probabilities of the particle’s path are indicated. B) Mean square displacement vs. time plots for EB1-NG particles with a ratio of <4 for the total distance moved to the end-to-end distance. Note that data follow not a linear but a parabolic path indicative for directed movements.

2.15 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), _ift20-1_ 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 (gcacacggtctagattcgcactgccgtgagc) and (gtctagaccagcactgccgctgagc), 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 the plasmid pCrEB1 (Zhu et al., 2013). 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 (gcaagaccggtgacatgaagcacagcg) and (ccagagcgactgacccaggcatcg) 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 XhoI site into which a 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 (taggtacctcagctgctaccaggc). 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 based on the NG protein sequence, using the Chlamydomonas codon bias, and amplified from the pBR25-CrNG plasmid using the primer pair (ctcgagatggtgctgagatgc) and (ctcgagctgtgctgctgctgct) 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 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 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 antiIC78 (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 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, we placed cells on ice until needed. Long-short cells were generated by passing cells in M media 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 DualView2). 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 on 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 (Bioptechs). 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 \( \sim 66 \) 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 \( \sim 20\% \) of the cell's total tubulin, corresponding to an \( \sim 12 \) times higher concentration in the flagellar compartment compared with the accessible cytoplasm (\( \sim 20\% \) of the total cell volume). In steady-state flagella, \( \sim 10\% \) of the tubulin is soluble; the share of soluble tubulin in the \textit{C. reinhardtii} cell body is unknown but has been estimated to be \( \sim 40\% \) or more in other cells. Assuming that \( \sim 60\% \) 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-\( \alpha \)-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, dx, from a Gaussian distribution with SD, \( \sigma = \sqrt{2D\Delta t} \), where D is the diffusion constant and \( \Delta t \) is the time step. For our simulations \( \Delta t = 0.1 \) s. D is position dependent:

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

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 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 tip dwell times. Simulations using a D(x) of
1.06 μm$^{-2}$ 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 $\text{NA}/\lambda$, 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

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

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

$$\text{PSF}(x, y) = |\mathcal{F}\{P(\mu, \nu) \exp(2\pi j(\mu x + \nu y))\}|^2$$

where $\mathcal{F}\{}$ 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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CHAPTER 3

DIFFUSION RATHER THAN IFT PROVIDES MOST OF THE TUBULIN
REQUIRED FOR AXONEMAL ASSEMBLY


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3.1 ABSTRACT

Tubulin enters the cilia by diffusion and motor-based intraflagellar transport (IFT). The respective contributions of each route in providing tubulin for axonemal assembly are unknown. To attenuate IFT-based transport, we expressed modified GFP-tubulins in strains possessing IFT81 and IFT74 with altered IFT-based tubulin binding sites. E-hook deficient GFP-β-tubulin normally incorporated into the axonemal microtubules; its transport frequency was reduced by ~90% in control cells and essentially abolished when expressed in a strain possessing IFT81 with an incapacitated tubulin-binding site. Despite the strong reduction in IFT, the share of E-hook deficient GFP-β-tubulin in the axoneme was only moderately reduced indicating that most axonemal tubulin (~80%) enters cilia by diffusion. While not providing the bulk of axonemal tubulin, we propose that motor-based IFT is nevertheless critical for ciliogenesis because it ensures high concentrations of tubulin near the ciliary tip promoting axonemal elongation.

3.2 INTRODUCTION

Tubulin is the main protein of the axoneme, the structural core of cilia and flagella (terms used interchangeably; [95]). The axonemal microtubules provide the scaffold onto which dynein arms and other complexes serving in ciliary motility assemble and serve as tracks for intraflagellar transport (IFT). During ciliary assembly, vast amounts of tubulin move from the cell body into the organelle, e.g., a 12-μm long axoneme contains ~350,000 tubulin dimers [96]. Tubulin moves inside cilia by diffusion and intraflagellar transport (IFT; [56, 84]). In IFT, arrays of IFT particle (= IFT trains) travel bidirectionally along the axonemal microtubules by the means of motor proteins [70, 97]. While IFT speed
and frequency remain essentially unaltered, the frequency of tubulin transport is upregulated while cilia elongate and attenuate in non-growing cilia. Similar to other small soluble proteins, tubulin continuously diffuses into cilia apparently moving freely through the ciliary gate formed by the transition zone at the ciliary base [84, 98, 99]. The respective contributions of diffusion and IFT in supplying tubulin for axonemal assembly are unknown. Manipulating IFT-tubulin interactions could help to estimate the share of tubulin supplied by each path.

In vitro interaction studies revealed that the N-terminal domains of the IFT particle proteins IFT81 and IFT74 form a bipartite tubulin-binding module (Bhogaraju et al., 2013). In detail, the calponin homology (CH)-domain of IFT81 binds to the globular core of the αβ-dimer providing specificity and the basic N-terminus of IFT74 interacts with the acidic C-terminal E-hook of β-tubulin stabilizing the interaction. In vivo, the deletion of the N-terminal 130 residues of IFT74 (IFT74Δ130) severely decreased the speed of ciliary regrowth; ciliary length, however, was only moderately reduced [100]. Mutations of two or five basic residues in the CH-domain of IFT81 diminishes tubulin binding in vitro [90]. However, C. reinhardtii expressing an altered IFT81 with five such point mutations (IFT81-5E) still assembles near full-length flagella albeit at a reduced rate (Kubo et al., 2016). In both the IFT81-5E and IFT74Δ130 strains IFT transport of GFP-α-tubulin was reduced potentially explaining the delay in ciliary growth. Double mutants in the tubulin binding sites of IFT81 and IFT74 assemble none or severely truncated flagella with an otherwise normal ultrastructure supporting the notion that the IFT81/74 module promotes ciliogenesis by facilitating IFT transport of tubulin (Kubo et al., 2016). While great care was taken to ensure that the above-described manipulations
of IFT81 and IFT74 are specifically affecting tubulin transport, defects in IFT itself cannot be excluded. The strain expressing the N-terminally truncated IFT74, for example, displayed anomalies in IFT speed and frequency and the abundance of various IFT proteins in the cell body; further, phospholipase D was enriched in cilia indicative for defects in retrograde IFT [80, 100]. Due to the short length of the cilia, IFT could not be assessed in the double mutant. It is therefore of interest to analyze how changes in tubulin itself affect its transport by IFT. Here, we focused on the E-hook of β-tubulin, which has been predicted to interact with the N-terminal domain of IFT74.

In the ciliate *Tetrahymena thermophila*, genomic replacement of wild-type β-tubulin with versions lacking a functional E-hook severely impairs cell viability and ciliary assembly [101, 102]. Thus, approaches altering the entire tubulin pool of a cell are unsuited to determine the specific effect of such manipulations on tubulin transport by IFT. Here, we expressed GFP-tagged α- and β-tubulins in *C. reinhardtii* to levels of 5-10% of the total tubulin and analyzed how the altered E-hooks affect IFT transport without perturbing either IFT itself, IFT of the endogenous tubulin, or ciliary assembly. This approach has the added potential to shed light on the relative contributions of IFT and diffusion in ciliary tubulin supply by analyzing how a reduction in IFT of a specific assembly-competent GFP-tagged tubulin will affect its share in the axoneme. We show that the deletion of the E-hook of β-tubulin severely reduced its ability to bind to IFT trains; the effect is specific for the E-hook of β-tubulin. IFT transport of E-hook-deficient β-tubulin was nearly abolished in a strain expressing IFT81 with defects in its tubulin-binding CH-domain. Nevertheless, E-hook deficient β-tubulin remained abundant in the axoneme suggesting a smaller than anticipated role of IFT in providing
tubulin for axonemal assembly. Based on our observations, we estimate that about 20% of the total tubulin in the axoneme is provided by IFT while ~80% enter cilia by diffusion. We postulate that the elongated geometry of cilia with just a narrow opening to the cell body delays tubulin entry by diffusion requiring tubulin transport by IFT to ensure ciliary assembly.

RESULTS

3.3 THE E-HOOK OF β-TUBULIN PROMOTES TUBULIN-IFT INTERACTION IN VIVO

SuperfolderGFP (GFP) was fused to the N-terminus of β-tubulin and expressed in wild-type Chlamydomonas reinhardtii (Fig. 1). Western blotting using anti-GFP and anti-β-tubulin confirmed expression and the presence of GFP-β-tubulin in flagella (Fig. 1A). Live cell imaging showed that GFP-β-tubulin is incorporated into both cytoplasmic and axonemal microtubules (Fig. 1B). As previously reported for GFP-α-tubulin, IFT of GFP-β-tubulin was rarely observed in steady-state flagella but occurred with high frequency in elongating flagella obtained by first deciliating the cells via a pH shock. (Fig. 1C) [84]. Therefore, the transport frequencies for all tubulin constructs used in this study were obtained using regenerating flagella with a length of ~4 – 9 µm. A comparison of strains expressing different amounts of tagged β-tubulin revealed an approximately linear correlation between the total amount of GFP-β-tubulin expressed, the frequency of its transport in regenerating flagella by IFT, and its share in the axoneme (Fig. 1D, E).

To investigate the role of the βE-hook for IFT of tubulin, GFP-tagged β-tubulin was modified by deleting of the C-terminal 13 residues (ΔE-hook), replacing five
glutamic acid residues in the E-hook with alanine undermining charged-based interactions (-5E-hook), or substituting the E-hook of β-tubulin with that of α-tubulin (αE-hook; Fig. 2, Table S1). Cells expressing the transgenic GFP-tubulins grew normally, showed wild-type motility, assembled full-length flagella at normal rates, and IFT of tubulin progressed at standard velocities (Table S1, not shown). To determine whether the E-hook modifications affected the frequency of GFP-β-tubulin transport, we selected strains expressing similar amounts of the transgenes as determined by Western blotting of whole cell samples (Fig. 2A). For all four constructs, ~8% of the total GFP-tagged protein was in the flagella and ~90% of the flagellar GFP-tubulin was in the axonemal fraction indicating that the fusion proteins entered the flagella and were assembly competent (Fig. 2B). All three mutations in the βE-hook resulted in significant reductions in the frequency of anterograde tubulin IFT with the E-hook deficient construct being the most affected (2.4 events/min, SD 2.9 events/minute, n=98 flagella analyzed compared to 21 events/minute, SD 14.3 events/minute, n=85 for wild-type β-tubulin; Fig. 2C).

Loss of the βE-hook weakens the interaction of tubulin with IFT in vitro [90] and we wondered how loss of the βE-hook will affect the processivity of tubulin transport. The axonemal protein DRC4-GFP, for example, frequently (~40%) dissociates from IFT trains before arriving at the flagellar tip [85]. In contrast, IFT transport of GFP-α-tubulin mostly (~98%) proceeds in one run to the tip indicative for a stable interaction with IFT trains [84]. Similarly, 97% (n= 559) of the transports involving wild-type GFP-β-tubulin proceeded nonstop to the tip of the elongating flagella whereas such processive IFT transports were reduced by 25-45% for the constructs with modified E-hooks (Fig. 2D
and E). In those strains, GFP-β-tubulin was observed converting from IFT transport to diffusion indicative for a dissociation from IFT (Fig. 2D).

Considering the apparent importance of the βE-hook for tubulin binding to IFT, we transplanted the E-hook and neighboring regions of β-tubulin onto GFP or mNeonGreen (mNG; Fig. S1). Similar to GFP alone, the GFP/mNG-βΔE-hook fusion proteins readily entered flagella by diffusion. In regenerating flagella, IFT transport of GFP alone was not observed. Anterograde IFT was observed for the GFP/mNG-βE-hook fusions albeit at extremely low frequencies (<0.05 events/min) indicating an almost complete absence of binding to IFT trains (Fig. S1). In summary, the βE-hook is neither sufficient nor necessary for IFT transport; its loss, however, reduces the frequency and processivity of tubulin transport supporting the view that the βE-hook stabilizes IFT-tubulin interactions.

3.4 HIGH FREQUENCY TRANSPORT OF TUBULIN REQUIRES THE E-HOOK OF β-TUBULIN TO INTERACT WITH IFT74

Bhogaraju et al. (2013) proposed that tubulin binding by IFT trains involves the CH-domain of IFT81 and an interaction between the E-hook of β-tubulin and the N-terminal domain of IFT74 (Fig. 3A). To further investigate the validity of this model, we analyzed tubulin transport in ift81-1 IFT81-5E, a strain in which five basic residues (K73, R75, R85, K112 and R113) critical for tubulin binding by the IFT81 CH-domain were replaced by glutamates, and in ift74-2 IFT74ΔN1-130, an IFT74 null mutant rescued with a version of IFT74 lacking the proposed N-terminal tubulin-binding domain (Fig. 3B-G); [96, 100]).
E-hook deficient β-tubulin is predicted to be unable to interact with the N-terminal domain of IFT74 and thus its binding to IFT trains should depend on the CH-domain of IFT81. In the *ift81-1 IFT81-5E* background, intermediate transport frequencies were observed for full-length β-tubulin while anterograde IFT of ΔE-hook β-tubulin was essentially abolished (<99% compared to full-length β-tubulin in strains with wild-type IFT81; Fig. 3B, C). Sporadic transport of E-hook deficient β-tubulin in *ift81-1 IFT81-5E* could be due to residual binding by IFT81-5E or low capacity binding elsewhere on IFT. The *ift74-2 IFT74ΔN1-130* strain lacks the proposed βE-hook-binding site of IFT74. The anterograde IFT frequencies of full-length and E-hook deficient β-tubulin were similarly low suggesting that both bind with similar strength to IFT trains presumably via the remaining IFT81-CH site (Fig. 3F). Thus, the lack of the N-terminal region of IFT74 renders IFT unable to discriminate between full-length and E-hook deficient β-tubulin.

We wondered whether IFT transport specifically requires the E-hook of β-tubulin or whether the E-hook of α-tubulin also contributes to IFT. In wild-type IFT cells, transport of E-hook deficient α-tubulin was somewhat reduced but still occurred at a high frequency when compared to E-hook deficient β-tubulin (Fig. 3D, G). Also, strains expressing *ift74-2 IFT74ΔN1-130* or *ift81-1 IFT81-5E* transported full-length and E-hook deficient α-tubulin with similar intermediate frequencies (Fig. 3D, G). Thus, the IFT system is largely insensitive to the loss of the αE-hook. Further, substitution of the E-hook of GFP-β-tubulin with that of α-tubulin did not restore normal transport frequencies in wild-type (Fig. 2C) or the *ift81-1 IFT81-5E* background (Fig. 3E). The data support the model by Bhogaraju et al. (2013) in which the E-hook of β-tubulin interacts with the
N-terminal domain of IFT74. We further show that loss of βE-hook in combination with mutations in the IFT81-CH domain severely reduce IFT transport of GFP-tubulin.

### 3.5 NEAR ABROGATION OF IFT OF TAGGED β-TUBULIN ONLY MODERATELY REDUCES ITS AXONEMAL PRESENCE

In addition to IFT, tubulin enters flagella also by diffusion. The expression of a tagged assembly-competent tubulin with strongly decreased binding to IFT facilitates an assessment of the respective contributions of IFT and diffusion in supplying axonemal tubulin. If, for example, ~90% of the flagellar tubulin were delivered by IFT, one would expect that a strong reduction in IFT of a specific tubulin species will be reflected by an almost equally strong reduction of its share in the axoneme. Toward this end, we analyzed the correlation between the frequency of IFT and the axonemal share for full-length and E-hook deficient GFP-β-tubulin (Fig. 4). Loss of the βE-hook reduced the frequency of IFT by ~90% in wild-type, the ift81 IFT81 rescue strain (expressing a wild-type IFT81 transgene), and the ift81 IFT81-5E rescue strain, each in comparison to full-length GFP-β-tubulin (Figs. 4A). Western blot analyses of axonemes, however, documented only a moderate reduction of E-hook deficient GFP-β-tubulin compared to the full-length version (Figs. 2A, 4B). Quantitative analysis of western blots showed a reduction of in average ~10% (SD 33%, n = 9) for truncated vs. full-length GFP-β-tubulin from nine independent flagella isolates (four, three, and two in the wild-type, ift81 IFT81, and ift81 IFT81-5E backgrounds, respectively; Figs. 4C, S2A). Western blots of isolated axonemes using anti-β-tubulin confirmed that full-length and truncated GFP-β-tubulin were present in similar ratios to the endogenous β-tubulin; for this experiment we
used a polyclonal anti-*Chlamydomonas*-β-tubulin since most commercial antibodies to β-tubulin react with the E-hook (Fig. 4D; [103]). Even in the near absence of transport by IFT, as observed for E-hook deficient GFP-β-tubulin in the *ift81 IFT81-5E* strain (~99.8% reduction in frequency in comparison to GFP-β-tubulin in wild-type), the truncated GFP-tagged tubulin was still well presented in the flagella (Fig. 4E; ~50% of the wild-type control, n=2 independent isolates). The average reduction of E-hook deficient GFP-β-tubulin’s share in the axoneme was 17% (SD 35%; n=10 flagellar isolates). In live cell imaging, the flagella of strains expressing full-length GFP-β-tubulin (in the *ift20 IFT20-mCherry* background to facilitate identification) and E-hook deficient GFP-β-tubulin were of similar brightness (Fig. 4F). In summary, the strong reduction in the frequency of IFT transport observed of E-hook deficient β-tubulin is not matched by a proportional reduction of its presence in the axoneme.

Our data suggest a limited quantitative contribution of IFT in providing tubulin for axonemal assembly. However, cells with defects in the IFT81/74 tubulin-binding module largely fail to assemble flagella. We therefore searched for possible alternative mechanisms potentially explaining the surprisingly low effect the reduction in the transport frequency of truncated β-tubulin has on its axonemal share. Mutations in IFT81N or IFT74N reduce IFT of tubulin and slow down flagellar growth suggesting that reduced IFT of tubulin could lower the rate of flagellar elongation [96]. Here, we used regenerating flagella to determine the IFT frequency while flagella assembled after the previous cell division were used for our biochemical analysis. The former requires approximately 90 minutes to regenerate flagella of 12-µm length whereas post-mitotic assembly is considerably slower completing ~ 8-µm long flagella in ~4 hours ([104, 105];
Postmitotic flagellar assembly could involve less IFT and more diffusion of precursors, potentially explaining the high share of ΔE-hook GFP-β-tubulin in flagella despite its low frequency of IFT. Western blot analysis, however, did not reveal a significant difference in the share of full-length and E-hook deficient β-tubulin in postmitotic vs. regenerated axonemes (Fig. S2B). Further, a strong discrimination of truncated GFP-β-tubulin by retrograde IFT could enrich it relative to the full-length protein. The frequencies of retrograde tubulin traffic, however, were negligibly low compared to anterograde traffic and were similar in range for full-length GFP-β-tubulin and its derivates (Fig. S2C, D). Also, the total amount of GFP-β-tubulin tolerated in axonemal microtubules could be limited and even low IFT frequencies as observed for truncated GFP-β-tubulin could still be sufficient to saturate GFP-tubulin incorporation. We consider this situation unlikely because the IFT frequency and axonemal quantity of full-length GFP-β-tubulin correlated well over a wide range of expression levels (Fig. 1D, E). Finally, E-hook deficient β-tubulin could incorporate into the axoneme at a significantly higher rate than the full-length protein. Of note, subtilisin-treated E-hook deficient tubulin has an about 50X lower critical concentration for polymerization into rings and sheets albeit assembly of bona fide microtubules was not observed [106, 107]. If E-hook deficient β-tubulin indeed incorporates into axonemal microtubule at a much higher rate than the full-length protein, one would expect the truncated version to be more abundant in the axoneme than full-length GFP-β-tubulin under conditions when both proteins show the same frequency of IFT. This is the case in the ift74-2 IFT74ΔN1-130 background when tubulin transport relies solely on the IFT81-CH. Comparison of the axonemes revealed similar shares for full-length and truncated
GFP-β-tubulin expressed at similar levels in *ift74-2 IFT74ΔN1-130* (Fig. S2E). In conclusion, our observations are explained best by assuming that IFT transports only a fraction of the tubulin present in the axoneme and that most tubulin enters flagella by diffusion.

**DISCUSSION**

Tubulin enters cilia by diffusion and as a cargo of IFT. The latter is regulated in a ciliary-length dependent manner and this regulation is likely to contribute to ciliary length control [56, 84, 96]. To explore the respective contributions of each route in providing tubulin for axonemal assembly, we manipulated tubulin transport by IFT taking advantage of the detailed knowledge of IFT-tubulin interactions. Previously *in vitro* studies showed that the E-hook of β-tubulin confers stable binding of tubulin dimers to the IFT81N/74N module by interacting with IFT74 [90]. In agreement with this model, removal of the E-hook of GFP-tagged β-tubulin reduced IFT by ~90%. However, the strong reduction in IFT was not mirrored by a comparative reduction of the share of E-hook deficient β-tubulin incorporated into the axoneme. We propose that IFT’s predominate role is to regulate intraciliary tubulin concentrations rather than providing the bulk of tubulin needed for ciliary assembly.

3.6 IFT74 SPECIFICALLY INTERACTS WITH THE β E-HOOK TO PROMOTE TUBULIN IFT

Bhogaraju and colleagues (2013) showed that tubulin-binding by the IFT81N/IFT74N module was significantly reduced when the E-hook of β-tubulin was removed by a brief
subtilisin treatment. Subtilisin treatment only mildly affected tubulin binding by IFT81N alone suggesting that the positively charged IFT74N interacts with negatively charged E-hook of β-tubulin. Our in vivo observations are in agreement with this model and provide the additional insights: 1) The E-hook of β-tubulin is not essential for binding to IFT trains or incorporation into the axonemal microtubules; it is also not sufficient for IFT transport. 2) IFT74N’s interaction with tubulin is specific for the β-tail while the negatively charged E-hook of α-tubulin is expendable. 3) One of the two predicted tubulin-IFT contacts (i.e., IFT81-CH and tubulin dimers, IFT74N and βE-hooks) is sufficient to maintain intermediate IFT transport frequencies and, as recently shown by Kubo et al. (2016), ciliary assembly. 4) GFP-tubulin transport was essentially abolished when the deletion of the βE-hook was combined with mutations in IFT81N, an arrangement thought to torpedo both interactions of the IFT81N/IFT74N module with tubulin. This suggests that IFT81N/IFT74N module is the major tubulin binding site on anterograde IFT trains and argues against the presence of additional tubulin binding sites independent of the IFT81N/IFT74N module [10]. This statement is supported by the recent finding from Kubo et al. (2016) that double mutants in IFT81N and IFT74N are essentially unable to assemble flagella.

3.7 THE β E-HOOK IN TUBULIN TRANSPORT AND MICROTUBULE STABILITY
Tubulin E-hooks are subjected to a variety of posttranslational modifications, which are thought to be critical for the stability of axonemal microtubules. After genomic replacement of the sole conventional β-tubulin gene of *Tetrahymena thermophila* with versions carrying modified E-hooks, cells assemble shorter than normal cilia frequently
lacking the ciliary central pair and the B-tubules of the doublets [101, 109-111]. The mutant phenotype has been explained by the lack of posttranslational modifications, in particular polyglycylation, which occurs on five sites in the *T. thermophila* βE-hook. Both the decrease in tubulin IFT in *C. reinhardtii* and the structural defects in the cilia of *T. thermophila* are specific for changes to the βE-hook raising the question whether reduced IFT of tubulin could contribute to the axonemal defects observed in the *T. thermophila* mutants. Removal of the E-hook from the sperm-specific β2-tubulin inhibits flagellar development in *Drosophila melanogaster*, and its replacement with the β1 E-hook obstructs central pair assembly [112]. *Drosophila* sperm flagella, however, assemble independently of IFT in the cytoplasm; diminished tubulin transport is therefore unlikely to contribute to the fly phenotype. We conclude that the E-hook of β-tubulin is critical for both high frequency IFT of tubulin and the stability of axonemal microtubules.

3.8 THE BULK OF AXONEMAL TUBULIN IS SUPPLIED BY DIFFUSION

Here, we circumvented problems in axonemal assembly caused by mutations in βE-hook by co-expressing GFP-tagged tubulin in cells possessing wild-type endogenous tubulins. This approach allowed us to analyze IFT transport of modified tubulins independently of ciliary assembly. Surprisingly, a strong reduction in IFT of E-hook deficient β-tubulin was not matched by a proportional reduction of its share in the axoneme. After assessing several possible mechanisms, we conclude that most of the tubulin in the axoneme enters the cilium by diffusion.

Estimates of the transport capacity of IFT support this notion: While several other IFT proteins possess CH-domains, only the ones of IFT81 and IFT54 bind tubulin
Recently, it has been shown that the CH-domain of IFT54 is expendable for ciliary assembly [113]. Thus, it is likely that the IFT81N/IFT74N module forms or is part of the only high capacity tubulin binding side of IFT. Ultrastructural data indicate that each IFT train contains about 40 IFT-B complexes and approximately 60 IFT trains enter the cilium each minute. The oligomeric state of tubulin during transport is unknown but the low stability of tubulin protofilaments suggests that tubulin is transported as a dimer. Under these assumptions, IFT could transport approximately 25% of the ~10,000 tubulin dimers required each minute during phases of rapid ciliary growth (~350 nm/minutes; [108]). Apparently, the IFT pathway simply lacks the capacity to be the major provider of axonemal tubulin during ciliary assembly. On the other hand, FRAP analysis revealed a continuous high-frequency influx of GFP-tubulin into cilia by diffusion [84]. The experimental data presented here suggest that roughly 4/5 of the axonemal tubulin enter the cilium by diffusion.

3.9 CILIARY GEOMETRY DELAYS DIFFUSION EXPLAINING THE NEED FOR TUBULIN TRANSPORT BY IFT

The above notion needs to be reconciled with observations indicating a critical role of IFT in providing tubulin for ciliary assembly, e.g., defects in the IFT81N/IFT74N module reduce the ciliary growth rate or impair ciliogenesis [90, 96]. Apicomplexan parasites such as Plasmodium rapidly assemble numerous axonemes within the cell body without IFT [114, 115]. In that situation, diffusion is sufficient to provide the tubulin necessary for axonemal assembly as it can be generally assumed for other large-scale microtubule-based assemblies such as the mitotic spindle. Compared to the open space of the cell
body cytoplasm, tubulin entering the cilium by diffusion will have to pass through the transition zone (TZ), a narrow opening between the cell body and the cilium [116]. The opening into the cilium corresponds to just ~0.01% of the surface of the plasma membrane and is further constricted by the TZ fibers [117]. Thus, the transition zone is a passive barrier slowing down the diffusional entry of soluble proteins into the cilium (Fig. 5). Given enough time, cell body and ciliary tubulin will equilibrate by diffusion. However, the physical barrier of the TZ could render diffusion insufficient to replace tubulin depleted from the ciliary matrix during rapid axonemal elongation in a timely manner. A transient decrease in the concentration of soluble tubulin could trigger depolymerization of the axonemal microtubules. We noted previously that the concentration of soluble tubulin inside growing cilia exceeds that of non-growing cilia even when both are present on the same cell body; the increase correlates with high frequency transport of tubulin by IFT into the growing cilium [84]. Rather than having an auxiliary role in tubulin delivery, motor-based IFT could maintain the ciliary concentration of tubulin above a threshold critical for efficient axonemal assembly. IFT unloads tubulin mostly near the ciliary tip, where the tubulin concentration is particularly critical for ciliary growth and where a high tubulin concentration will be maintained longer since dilution by diffusion is most limited near the ciliary tip [118]. Even with the bulk of axonemal tubulin entering cilia by diffusion, ciliary assembly could still be incapacitated in the absence of functional IFT because the intraciliary tubulin concentration will not reach the level necessary for assembly by diffusion alone. We propose that IFT regulates the intraciliary concentration of tubulin to promote axonemal assembly and cilia elongation.
Figure 1

A. whole cell

B. GFP-fluorescence

C. regenerating steady state

D. kDa

E. bar graph
Figure 1: GFP-β-tubulin is transported by IFT and incorporated into microtubules.

(A) Western blots analyzing whole cells and isolated flagella from a wild-type strain expressing GFP-β-tubulin and an untransformed control strain (WT). Western blots were stained with a polyclonal antibody to β-tubulin and anti-GFP; the position of endogenous β-tubulin, the GFP tagged version, and marker proteins are indicated. (B) Life cell imaging of a cell expressing GFP-β-tubulin in DIC (a) and TIRF (b, d, e); an overlay image is shown in c. The TIRF images present a focal series showing the level of the cilia (marked by arrowheads in b), the basal bodies (arrow in d), and a more posterior section showing the cortical microtubules of the cell body (arrowheads in e). Bar = 10 μm. (C) Kymograms showing IFT transport (arrowheads) of GFP-β-tubulin in steady-state (a) and regenerating (b) flagella. The tip and the base of the cilia are indicated. Bars = 2 s and 2 μm. (D) Western blot of whole cell and axoneme samples obtained from four wild-type strains (Nos. 1 – 4) expressing different levels of GFP-β-tubulin. Blots were stained with anti-GFP; antibodies to the cell body protein NGFI-A binding protein 1 (NAB1) and the axonemal dynein intermediate chain 2 (IC2) were used as loading control for the cell body and axonemes, respectively. (E) Frequency of GFP-β-tubulin anterograde transport during flagellar regeneration in the strains shown in Fig. 1D.
Figure 2

A

<table>
<thead>
<tr>
<th></th>
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<tr>
<td>wt E-hook</td>
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</tr>
<tr>
<td>αE-hook</td>
<td>anti-NAB1</td>
</tr>
<tr>
<td>-5E-hook</td>
<td>anti-GFP</td>
</tr>
<tr>
<td>ΔE-hook</td>
<td>anti-IC2</td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th></th>
<th>anti-GFP</th>
</tr>
</thead>
<tbody>
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<td>wt E-hook</td>
<td></td>
</tr>
<tr>
<td>αE-hook</td>
<td></td>
</tr>
<tr>
<td>-5E-hook</td>
<td></td>
</tr>
<tr>
<td>ΔE-hook</td>
<td></td>
</tr>
</tbody>
</table>

C

![Bar graph showing the distribution of a quantifiable measure across different conditions.](image)

D

![Images showing distribution of GFP-β-tubulin across different conditions.](image)

E

![Graph showing the percentage of base-to-tip transports across different conditions.](image)
Figure 2: The E-hook of β-tubulin promotes transport by IFT.

(A) Western blot analyses of whole cells (WC), isolated flagella (FLA), and axonemes (AX) from strains expressing GFP-β-tubulin with wild-type and modified E-hooks. Anti-GFP was used to visualize the tagged β-tubulins and antibodies to NAB1 and IC2 as loading controls for WC and FLA/AX, respectively. (B) Left side: Western blots comparing the amount of GFP-β-tubulin in the deflagellated cell bodies (CB) to that in flagella (FLA; left) and (right side) comparing its level in the axonemes (AX) to that in the detergent soluble membrane+matrix fraction (MM, right) using a dilution series. 100% indicates that equivalents of the two fractions were loaded (i.e., one CB per two FLA etc.). (C) Histogram showing the average frequency of anterograde IFT events observed for the GFP-tagged β-tubulins. The number of regenerating cilia analyzed for each strain (n) and the standard deviation are indicated. (D) Kymograms showing transport of full-length and E-hook deficient (ΔE-hook) β-tubulin in regenerating flagella. Selected tracks are marked on the duplicates (bottom panels, respectively). Note the reduced frequency and reduced run length of transports involving the truncated GFP-β-tubulin (blue lines). Bar = 1s 1μm. (E) Histogram comparing the share (in %) of early-terminating transport events of wild-type and modified GFP-β-tubulins. The number of processive base-to-tip events and the total number of events analyzed are indicated.
Figure 3: Transport of E-hook deficient β-tubulin is sensitive to mutations in IFT81.

(A) Schematic presentation of tubulin binding by the IFT81N/IFT74N module (modified after [90]). (B) Western blot comparing the amounts of wild-type and ΔE-hook GFP-β-tubulin in the ift81-1 IFT81 and the ift81-1 IFT81-5E rescue strains. The transgene expression levels were similar in all four strains. For this blot, loading was adjusted to result in anti-GFP signals of similar strength. (C) Histogram showing the average frequencies of anterograde IFT events observed for full-length (wt) and truncated (Δβ) GFP-tagged β-tubulins in cilia of the ift81-1 IFT81 and the ift81-1 IFT81-5E strains. The number of regenerating cilia analyzed for each strain (n) and the observed standard deviation are indicated. (D) Frequency of anterograde IFT for full-length and E-hook deficient GFP-α-tubulin in the ift81-1 IFT81 and the ift81-1 IFT81-5E strains. The corresponding western blots of the whole cell samples of these strains are shown below the graph. (E) Anterograde transport of αE-hook-GFP-β-tubulin (see D for details). (F, G) Anterograde transport of full-length and E-hook deficient GFP-β-tubulins (F) and GFP-α-tubulin (G) in the ift74-2 IFT74Δ130 strain and wild-type controls. Western blots of whole cell documenting similar expression of the transgenes are shown (bottom).
Figure 4

A

\[ \begin{align*}
\text{reduction in IFT} \\
(\text{wt E-hook} = 1)
\end{align*} \]

\[ \begin{align*}
\text{WT E-hook} & \quad \Delta E\text{-hook} \\
\text{wt} & \quad 1 \\
\text{ift81} & \quad 0.8 \\
\text{IFT81} & \quad 0.5 \\
\text{IFT81-5E} & \quad 0.2
\end{align*} \]

B

\[ \text{strain:} \quad \text{wild type} \quad \text{ift81} \quad \text{IFT81} \quad \text{IFT81-5E} \]

\[ \text{construct:} \quad \text{WT E-hook} \quad \Delta E\text{-hook} \]

\[ \begin{align*}
\text{whole cell} & \quad 75 \text{ kD} \\
\text{axonemes} & \quad 75 \text{ kD} \\
75 \text{ kD} & \quad \text{anti-GFP} \\
25 \text{ kD} & \quad \text{anti-NAB1} \\
75 \text{ kD} & \quad \text{anti-GFP} \\
75 \text{ kD} & \quad \text{anti-NAB1} \\
75 \text{ kD} & \quad \text{anti-GFP} \\
50 \text{ kD} & \quad \text{anti-IC2}
\end{align*} \]

C

\[ \begin{align*}
\text{axonemal incorporation} \\
(\text{wt E-hook} = 1)
\end{align*} \]

\[ \begin{align*}
\text{WT} & \quad n=4 \\
\text{ift81} & \quad n=3 \\
\text{IFT81-5E} & \quad n=2
\end{align*} \]

D

\[ \begin{align*}
\text{WT E-hook} & \quad \Delta E\text{-hook} \\
\text{axonemes} & \quad 75 \text{ kD} \\
75 \text{ kD} & \quad \text{anti-GFP} \\
75 \text{ kD} & \quad \text{anti-IC2} \\
50 \text{ kD} & \quad \text{anti-IC2}
\end{align*} \]

E

\[ \text{strain:} \quad \text{WT} \quad \text{ift81} \quad \text{IFT81-5E} \]

\[ \text{construct:} \quad \text{WT E-hook} \quad \Delta E\text{-hook} \]

\[ \begin{align*}
\text{WC} & \quad 75 \text{ kD} \\
25 \text{ kD} & \quad \text{anti-GFP} \\
\text{FLA} & \quad 75 \text{ kD} \\
75 \text{ kD} & \quad \text{anti-IC2}
\end{align*} \]

F

\[ \text{strains:} \quad \text{GFP-}\beta\text{-tubulin} \quad \text{IFT20-mCherry} \quad \text{GFP-}\Delta E\text{-hook-}\beta\text{-tubulin} \]

\[ \text{GFP} \quad \text{mCherry} \quad \text{merge} \]

\[ \begin{align*}
\text{anti-GFP} & \quad \text{anti-NAB1} \\
\text{anti-GFP} & \quad \text{anti-IC2} \\
\text{anti-IC2} & \quad \text{anti-IC2}
\end{align*} \]
Figure 4: Abated IFT of E-hook deficient tubulin does not cause a proportional reduction of its presence in the axoneme.

(A) Histograms comparing the reduction in IFT transport of ΔE-deficient vs. full-length β-tubulin in wild-type, ift81-1 IFT81, and ift81-1 IFT81-5E rescue strains. The frequencies observed for the full-length GFP-β-tubulin in each background were set to 1. See Fig. 3 for the original data, SDs, and n values. (B) Western blot of wild-type and ift81-1 rescue strains comparing whole cell and axoneme samples using anti-GFP and antibodies to NAB and IC2, as loading controls. (C) Histograms comparing the changes in the axonemal amounts of ΔE-deficient vs. full-length β-tubulin in wild type, and the ift81-1 IFT81 and ift81-1 IFT81-5E rescue strains as deduced from anti-GFP stained Western blots. The band intensities of the full-length GFP-β-tubulin in each background were set to 1. n, number of independent flagella isolates. (D) Western blot comparing isolated axonemes from a wild-type strain expressing full-length or truncated β-tubulin stained with anti-GFP, anti-β-tubulin, and anti-IC2. (E) Western blot of whole cell (WC) and flagella (FLA) samples comparing strains expressing full-length β-tubulin in wild type vs. ΔE-deficient β-tubulin in the ift81-1 IFT81-5E strain side by side. The antibodies used are indicated. (F) TIRF images showing the GFP and mCherry signals in cilia of live cells. Cells expressing E-hook deficient β-tubulin and cells expressing full-length β-tubulin in the ift20-1 IFT20-mCherry background were mixed to allow for a comparison of the signal strength with the same microscope settings. Bar = 10 µm.
Figure 5

(a) no IFT

\[ C_{\text{cilium}} = C_{\text{cell body}} \]

(b) with IFT

\[ C_{\text{cilium}} > C_{\text{cell body}} \]
**Figure 5: Tubulin transport by IFT enables ciliary elongation.**

Schematic presentation of tubulin influx into cilia without (a) and with (a) IFT. (a) In the absence of IFT, tubulin (green dots) diffusing in the cell body (dashed lines) will eventually enter the cilium. Proteins entering cilia by diffusion will have to pass through the narrow openings of the transition zone at the ciliary base. This geometry will delay diffusional entry of tubulin into the cilium and axonemal elongation could decrease the concentration of soluble tubulin halting further elongation. (b) IFT trains will pick-up tubulin near the flagellar base, move it through the physical barrier of the transition zone, and release it near the ciliary tip. IFT increases the tubulin concentration in the cilium, particularly near the ciliary tip, above that of the cell body promoting axonemal elongation.
Figure S1

A

Control

GFP

GFP::βE-hook

GFP

Linker

βE-hook

(α-tubulin$^{398-431}$)

(β-tubulin$^{421-443}$)

crNeon::β-H11/12-E-hook

crNeon

H11

H12

βE-hook

(β-tubulin$^{370-443}$)

B

Control

GFP::βE-hook

GFP::βE-hook

GFP::βE-hook

crNeon::β-H11/12-E-hook

10 fps

30 fps

10 fps
Figure S1: The βE-hook is not sufficient to mediate transport of GFP by IFT.

(A) Schematic presentation of the construct fusing GFP or mNG to C-terminal regions of β-tubulin. (B) Kymograms of the constructs shown in A. Note that the associated videos were recorded at different frame rates (fps). Bars = 1s 2μm.
Figure S2

A

B

C

D

E
Figure S2: Transport and axonemal incorporation of E-hook-deficient β-tubulin.

(A) Western blot analysis of isolated axonemes of the ift81-1 IFT81 strain expressing full-length (WT) GFP-β-tubulin. The samples were diluted as indicated and compared to the undiluted sample of a strain expressing ΔE-hook βtubulin in the same background.

(B) Comparison of axonemes isolated from postmitotic and regenerated cilia and the corresponding whole cell samples. Regenerated cilia were isolated ~3 hours after the pH-shock; postmitotic cilia ~6 hours after the onset of the light. Antibodies to IC2 and NAB were used as loading controls.

(C) Kymogram and replicate kymogram with marked tracks showing retrograde IFT of E-hook-deficient β-tubulin. (D) Histogram showing the frequency of retrograde IFT for full-length (WT) and the modified β-tubulins in regenerating cilia.

(E) Analysis of the distribution of GFP-tagged full-length and truncated β-tubulin in whole cell (WC) and axonemes (AX) of the ift74-2 IFT74Δ130 strain. In this background, full-length and truncated tubulin are transported with similar frequencies by IFT and entry into the cilium will largely occur by diffusion. Both tubulin are incorporated in similar amounts into the axoneme suggesting that the incorporation of tubulin into the axoneme is not affected by the absence or present of the βE-hook. The strains selected for this experiment expressed GFP-tubulin at less than 2% of the total tubulin.
3.12 SUPPLEMENTARY TABLES

Table 1: Tubulin constructs used in this study.

<table>
<thead>
<tr>
<th>protein</th>
<th>variant</th>
<th>C-terminal sequence</th>
<th>anterograde IFT (µm/s)</th>
<th>retrograde IFT (µm/s)</th>
<th>Swimming (µm/s)est.</th>
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<tr>
<td>GFP-β-tubulin</td>
<td>WT E-hook</td>
<td>SAEEEGEFEGEAE*</td>
<td>2.2 (SD 0.5, n = 360)</td>
<td>2.45 (SD 1, n = 18)</td>
<td>86 (SD 24, n = 230)</td>
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<tr>
<td>GFP-β-tubulin</td>
<td>αE-hook</td>
<td>SAEAGAEGEAY*</td>
<td>2.13 (SD 0.44, n = 33)</td>
<td>2.7 (SD 0.5, n = 5)</td>
<td>75 (SD 19, n = 163)</td>
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<td>GFP-β-tubulin</td>
<td>-5E-hook</td>
<td>SAEAAAGAAGAAE*</td>
<td>1.95 (SD 0.49, n = 71)</td>
<td>2.8 (SD 1.3, n = 16)</td>
<td>70 (SD 21, n = 146)</td>
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<tr>
<td>GFP-β-tubulin</td>
<td>ΔE-hook</td>
<td>SA*</td>
<td>2.29 (SD 0.45, n = 50)</td>
<td>2.9 (SD 1.2, n = 69)</td>
<td>80 (SD 22, n = 235)</td>
</tr>
<tr>
<td>GFP-α-tubulin</td>
<td>WT E-hook</td>
<td>LEKDFEVEGAESAEGAGEEY*</td>
<td>2.09 (SD 0.35, n = 27)</td>
<td>n.d.</td>
<td>n.d.</td>
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<td>GFP-α-tubulin</td>
<td>ΔE-hook</td>
<td>LEKDFEVEGAEY*</td>
<td>1.91 (SD 0.35, n = 20)</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Table 1: Tubulin constructs used in this study.

Sequences of the E-hooks of wild-type GFP α- and β-tubulin and the modified tubulin molecules. Transport velocities of the fusion proteins by IFT and the swimming velocities of the transformants are listed.
3.13 MATERIALS AND METHODS

**Strains and culture conditions**

*C. reinhardtii* was maintained in modified M medium at room temperature, aeration with 0.5% CO₂, and a light/dark cycle of 14:10 h. Strain CC-620 was used for most experiments (http://www.chlamycollection.org/).

**Generation of transgenic strains**

The TUB2 gene encoding β-tubulin was synthesized omitting the second intron and introducing flanking BamH1 and EcoR1 sites (Genewiz). The TUA1 gene in the previously described vector pBR25-sfGFP-α-tubulin [84, 119] was replaced with the modified TUB2 using digestion with BamH1 and EcoR1 and ligation. Changes in the E-hook of β-tubulin were prepared as follows: gene segments encoding the modified C termini (E-hooks) of β-tubulin were synthesized (Genewiz), excised with EcoRI and EcoRV, and ligated into pBR25-sfGFP vector digested with the same enzymes taking advantage of a unique EcoRV site near the 3'-end of the TUB2 gene. Plasmids were restricted with KpnI and XbaI, and a fragment encompassing the functional Ble::GFP-β-tubulin cassette was gel-purified and transformed into *C. reinhardtii* by electroporation. TAP plates with 10 μg/ml zeocin were used to select transformants. Transformant colonies were transferred to liquid media in 96-well plates and screened by TIRF microscopy for expression of GFP. Expression of GFP-α-tubulin has been previously described [84]. The *ift20-1 IFT20-mCherry* strain was transformed with the construct encoding sfGFP-β-tubulin to obtain a double-tagged strain [81].

**Isolation and fractionation of cilia**
To isolate cilia for biochemical analysis we followed the protocol by [120]. Cells were concentrated by centrifugation (1,150 x g, 3 minutes, RT) and washed with 10 mM Hepes, pH 7.4. Cells were resuspended in HMS (10 mM Hepes, 5 mM MgSO4, and 4% sucrose) and deciliated by the addition dibucaine (final concentration of 4.17 mM; Sigma-Aldrich) and vigorous pipetting. The cell bodies were removed by centrifugation and cilia were sedimented (17,000 g, 4°C, 20 min). Isolated cilia were resuspended in a microtubule-stabilizing buffer, HMEK (30 mM HEPES, 5 mM MgSO4, 0.5 mM EGTA, and 25 mM KCl) plus protease inhibitor (P9599; 1:100; Sigma-Aldrich) and demembranated by addition of NP-40 Alternative (1% final concentration; EMD Millipore) on ice for 20 min. Axonemes were pelleted from membrane/matrix fraction by centrifugation (30,000 g, 4°C, 20 min) and fractions were analyzed by SDS-PAGE and Western blotting.

**Western blotting**

Ciliary proteins were separated by SDS-PAGE and transferred to PVDF membrane (Immobilon; Millipore) using standard protocols. The following primary antibodies were used: rabbit anti-Chlamydomonas-β-tubulin (1:2,000; [103], mouse anti-IC2 (1:50; [121], rabbit anti-GFP (1:500; Invitrogen), and rabbit anti-NAB1 (1:5,000; Agrisera). Western blots were developed using anti–mouse or anti–rabbit secondary antibodies conjugated to horseradish peroxidase (Molecular Probes) and chemiluminescence substrate (SuperSignal West Dura; Thermo Fisher Scientific). A ChemiDoc MP imaging system was used for imaging and Image Lab (both Bio-Rad Laboratories) was used for signal quantification via densitometry.
Ciliary regeneration

Cells were washed and resuspended in M media, deflagellated by a pH shock (pH ~4.2 for 45 s), transferred to fresh M medium, and allowed to regrow cilia under constant light with agitation [122]. To delay the onset on regeneration, cells were kept on ice until needed. To initiate regeneration, cells were diluted into ambient temperature M medium.

In vivo microscopy

For in vivo imaging of GFP-tubulin by through-the-objective TIRF illumination, we used a Nikon Eclipse Ti-U equipped with 60× NA1.49 objective and 40-mW 488-nm and 75-mW 561-nm diode lasers (Spectraphysics; [94, 123]). The excitation lasers were cleaned up with a Nikon GFP/mCherry TIRF filter and the emission was separated using an image splitting device (Photometrics DualView2 with filter cube 11-EM). Specimens were prepared as follows. Cells (~ 10 μl) were placed inside a ring of vacuum grease onto a 24x60mm No. 1.5 cover glass and allowed to settle (~1 – 3 minutes). The observation chamber was closed by inverting a 22×22 mm No. 1.5 cover glass with ~10 μl of 5 mM Hepes, 5 mM EGTA pH 7.3 onto the larger cover glass. Images were recorded at 10–31 fps using an iXON3 (Andor) and the NIS-Elements Advanced Research software (Nikon). ImageJ (National Institutes of Health) with the LOCI plugin (University of Wisconsin, Madison WI) and multiple kymogram plugin (European Molecular Biology Laboratory) were used to generate kymograms as previously described [94]. To photobleaching the cilia, the intensity of the 488-nm laser was increased to ~10% for 4 – 12 s.
**Video analysis**

IFT transport events were identified manually in ImageJ. The angle tool was used to determine the velocity of the transport events. Data were transferred to Excel for further analysis. If not stated differently in the figure legend, the n values states the number of cilia analyzed. Individual frames and kymograms were save in ImageJ, Adobe Photoshop was used to adjust brightness and contrast, and figure were mounted in Illustrator.
CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

4.1 SUMMARY OF DISSERTATION

Work contained in this dissertation begins to reveal the overall contributions of diffusion and IFT in providing proteins for ciliary assembly and maintenance. To investigate these two routes of protein transport in greater detail we focused on EB1 and tubulin. While these two proteins are both relatively small and display properties similar to one another (they both form dimers for example), their translocation into cilia varies greatly.

EB1 is present at the tips of cilia after IFT has been switched off in *Chlamydomonas fla10* cells, a temperature-sensitive mutant in the kinesin II motor [57]. Additionally, EB1 was shown to remain at the tip in non-growing and actively resorbing cilia [57]. Two scenarios could explain the above results: 1) EB1 is deposited at the ciliary tip via IFT where it remains firmly attached in this location when IFT is abolished; or 2) EB1 at the ciliary tip could be continuously exchanged in a manner independent of the IFT system. To distinguish between these two scenarios we expressed fluorescently-labeled EB1 protein in wild-type cells and examined its dynamics using *in vivo* imaging. EB1-FP (utilized both GFP and mNG fluorophores) displayed a cellular localization similar to the endogenous protein with an approximately equal distribution between the cytoplasm and ciliary compartments. EB1-FP accumulated in the distal tip
region of cilia confirming previous biochemical studies by Pederson et al [57]. In the cytoplasm, we observed EB1-FP comets that originated from the apical region of the cell and traveled posteriorly with a growth rate similar to that of cytoplasmic microtubules in plants [124]. These comets indicate that EB1-FP tracks the growing end of both cytoplasmic and ciliary microtubules and that EB1 in *C. reinhardtii* behaves similarly to EB1’s in other systems. Additionally, we showed that a subset of cortical microtubules in *Chlamydomonas* are highly dynamic and that the ciliary basal apparatus continuously nucleates cytoplasmic microtubules – a novel result in *C. reinhardtii*.

To elucidate the dynamics of EB1-FP at the ciliary tip, we examined the recovery of EB1-FP fluorescence following photobleaching in wild-type cells. Recovery of EB1-FP fluorescence at the tip was apparent briefly after photobleaching (within 10 seconds; however, full recovery required approximately 300 seconds) and recovery continued after repeated bleaching events at the tip. These results show that EB1 is not firmly attached to the ciliary tip. Instead, ciliary EB1 is continuously exchanged with EB1 from the rest of the cell. To determine if the recovery of EB1-FP fluorescence at the ciliary tip was dependent on IFT we analyzed the recovery dynamics of EB1-FP in *fla10-1* cells, which allows inactivation of IFT by incubating cells at a higher temperature. Our analysis showed that there was no significant difference in the rate at which EB1-FP recovered at the ciliary tip in the presence or absence of IFT. Additionally, two-color imaging experiments in which cells expressed both EB1-FP and IFT20-mCherry (a protein of the IFT-B2 complex) revealed that EB1-FP and the IFT particle move independently of one another with EB1-FP moving solely by diffusion. These results show that the location
and exchange processes of EB1 at the ciliary tip are not dependent on IFT, and that EB1 uses diffusion to enter and translocate within cilia.

To characterize the diffusional behavior of EB1-FP in cilia in greater detail we used higher laser intensities for our \textit{in vivo} imaging experiments. The higher laser intensity bleached most EB1-FP particles which prevented the accumulation of EB1-FP and enabled us to observe the movements of individual EB1-FP particles inside cilia. EB1-FP moved swiftly along the ciliary shaft with a one-dimensional diffusion coefficient of 1.06 μm²s⁻¹. Near the ciliary tip, however, this mobility was markedly reduced with a one-dimensional diffusion coefficient of 0.0063 μm²s⁻¹. EB1-FP frequently displayed periods of stationary behavior at the ciliary tip and dwelled in this region for an average of 2.5 seconds suggesting the presence of transient binding sites for EB1 at the tip of cilia. Simulations using the two distinct mobilities of EB1-FP in cilia showed that the diffusion and transient binding of EB1 were sufficient to explain its accumulation at the tip. From these results, we conclude that proteins that are equally distributed between the cytoplasm and cilia can rapidly concentrate at specific sites within the cilium independently of IFT by “diffusion and capture”. We showed that the presence of EB1 at the tips of steady-state cilia does not depend on the \textit{de novo} addition of tubulin to the axoneme. These results suggest that the presence of EB1 at the ciliary tip is not an indicator for the presence of GTP-bound tubulin. Instead, we propose that the tips of axonemal microtubules possess a lattice conformation that enables EB1 binding independently of the GTP state of tubulin. This lattice conformation would be permanent explaining how EB1 localizes to the tip of cilia regardless of the ciliary growth state. While the binding sites for EB1 in cilia are permanent, this is not necessarily always the
case. If the interaction between the diffusing protein and its binding sites were regulated, accumulation of the protein in cilia would occur only following the activation of the binding sites or protein. Upon activation, the protein would rapidly accumulate in cilia using diffusion. When the binding is abolished following inactivation, the protein would exit the cilium reestablishing the equilibrium concentration between cilia and the cytoplasm. Such a mechanism could explain how Gli proteins accumulate at the ciliary tip during hedgehog signaling [125].

In contrast to EB1, tubulin has been shown to enter cilia by diffusion and IFT [84]. Knowledge of how these two routes provide the tubulin necessary for axonemal assembly is unknown. To examine the respective contributions of each route in tubulin transport we attenuated the IFT transport of tubulin-GFP through the expression of modified GFP-tagged β-tubulin in strains that possessed IFT74 and IFT81 proteins with altered tubulin binding domains. Since the modified tubulin is expressed in cells that also contain wild-type tubulin, transport of the altered tubulin can be analyzed without affecting ciliogenesis. The importance of the E-hook of β-tubulin in IFT transport was addressed first, followed by how the tubulin binding domains of IFT74 and IFT81 influence tubulin transport by IFT.

To investigate the role of the βE-hook for IFT transport of tubulin, a GFP-tagged E-hook deficient β-tubulin was expressed in wild-type cells and its movements were examined with in vivo imaging. Western blot analysis showed that the altered construct was present in both cilia and axonemal fractions indicating E-hook deficient β-tubulin is transported into cilia and incorporated into the axoneme. IFT based transport of the E-hook deficient β-tubulin occurred at a significantly reduced rate. We observed an
approximately 90% reduction in IFT of E-hook deficient β-tubulin compared to that seen in cells expressing an unaltered GFP-tagged β-tubulin. The extreme reduction in IFT of E-hook deficient β-tubulin suggests that the E-hook is important for tubulin binding to the IFT particle. Considering this, we transplanted the E-hook of β-tubulin onto GFP to see if the E-hook alone was sufficient to drive IFT of an exogenous protein. However, like our GFP control, our β-tubulin E-hook fusion proteins readily entered cilia by diffusion and IFT-based transport of the construct was observed but only at extremely low frequencies. These results reveal that the E-hook of β-tubulin is neither necessary nor sufficient for IFT transport. However, loss of the E-hook significantly reduces the frequency of tubulin transport by IFT suggesting that this region is important in stabilizing the IFT-tubulin interaction for transport.

A model proposed by Bhogaraju et al. suggests that the binding of tubulin to IFT involves the CH-domain of IFT81 and an interaction between the E-hook of β-tubulin and the N-terminal domain of IFT74 [90]. To investigate the soundness of this model, we analyzed the transport of β-tubulin with and without the E-hook in an IFT81 stain in which five of the residues critical for tubulin binding were altered (IFT81-5E). Additionally, the transport of both β-tubulin constructs was examined in an IFT74 null mutant strain rescued with a version of the IFT74 protein that lacked the N-terminal domain (IFT74ΔN). For clarity, in the IFT74ΔN strain, where E-hook deficient β-tubulin was expressed, the interaction between the E-hook of β-tubulin and the N-terminal region of IFT74 is disrupted. However, the tubulin dimer can still interact with IFT through binding to IFT81. In contrast, in the IFT815E stain expressing E-hook deficient β-tubulin the interaction between IFT81 and the globular interface of the tubulin dimer is
abolished. Furthermore, the interaction between the E-hook of β-tubulin and IFT74 is disrupted in this strain. This situation disrupts all known binding of tubulin to IFT. However, because wild-type tubulin is present in these cells, ciliary assembly will occur. IFT-based transport of both full-length and E-hook deficient β-tubulin were similarly low in IFT74ΔN cells. These results suggest that the residue binding of both constructs to IFT is a result of interactions with IFT81. Our analysis of tubulin transport in the IFT815E background revealed that full-length β-tubulin was transported with intermediate frequencies, whereas IFT transport of E-hook deficient β-tubulin was effectively abolished being reduced by approximately ~99% when compared to the transport of full-length β-tubulin in wild-type cells. These data show that IFT based transport of tubulin is severely diminished when alterations of the E-hook of β-tubulin are paired with mutations in the tubulin binding domain of IFT81. Additionally, we show that IFT74 is able to discriminate between full-length and E-hook deficient versions of β-tubulin.

The manipulations of IFT and the E-hook of β-tubulin are unlikely to affect entry of tubulin-GFP into the cilia by diffusion. This scenario allows us to analyze the contributions of diffusion and IFT in providing tubulin for axonemal assembly. For example, if IFT provides 90% of the total tubulin needed for axonemal assembly then in wild-type cells expressing E-hook deficient β-tubulin (where IFT transport of E-hook deficient β-tubulin was reduced by ~90%) we should see an almost equal reduction in the presence of E-hook deficient β-tubulin in the axoneme. To examine this hypothesis, we analyzed the share of full-length and E-hook deficient β-tubulin in the axoneme of wild-type cells using Western blotting. Interesting, we observed only a moderate reduction in the amount of E-hook deficient β-tubulin present in axonemes compared to the full-length
version with quantitative analysis noting a reduction in average of ~10% for the E-hook deficient β-tubulin. For extreme cases, where IFT transport of tubulin was reduced to almost zero (IFT815E cells expressing E-hook deficient β-tubulin), the truncated version of β-tubulin was still well represented in flagellar fractions. On average, the reduction of E-hook deficient β-tubulin in axonemal fractions was only ~20%. Our data show that a strong reduction in the frequency of IFT based transport of E-hook deficient β-tubulin results in only a marginal reduction of its presence in the axoneme. We conclude that IFT contributes a limited amount of the total tubulin needed for axonemal assembly with diffusion providing the bulk of ciliary tubulin. While the contribution of IFT in providing ciliary tubulin may not be as high as some would have expected we posit that the share of tubulin supplied by IFT is vital to the overall growth of the cilium. Rather than taking a back seat to diffusion, we propose that IFT is utilized to regulate the intraciliary concentration of tubulin and ensure that high quantities of tubulin are present at the ciliary tip to promote axonemal assembly and therefore, ciliary growth.

4.2 TUBULIN TRANSPORT BY IFT AND ITS IMPACT ON CILIARY LENGTH REGULATION

Ciliary length is a tightly regulated process as the length of the organelle has a drastic effect on function. Proper ciliary length is vital to cell locomotion and signaling, as well as the ability to clear debris and propel fluid in the airways and brain, respectively. However, aberrations in ciliary length are not uncommon. Several *Chlamydomonas* mutants with defects in ciliary length regulation have been isolated; while others exist we will focus on those that assemble cilia that are too long [126-129].
An increase in ciliary length could result from a number of situations one of which is an increase in the amount of cargo transported by IFT trains. Just as the size of a skyscraper is dependent upon the amount of building material, the length of the cilium could be a reflection of the amount of tubulin, the main structural protein of cilia, provided.

In this dissertation, we posit that while a vast amount of tubulin utilizes diffusion for ciliary entry IFT transport of tubulin is essential for the polymerization of axonemal microtubules and therefore, elongation of the cilium. Such a theory assumes that the length of cilia is a reflection of the amount of tubulin provided by IFT: an increase in tubulin transport via IFT results in cilia of longer length while shorter cilia are caused by a lack of tubulin transport. What possible mechanisms could regulate the amount of tubulin and other axonemal proteins transported by IFT? Recent work from our lab begins to elucidate this issue by showing that mutants with longer than normal cilia display defects in the regulation of tubulin transport [84]. In wild-type cells, the amount of tubulin transported by IFT is tightly coupled to the growth state of the cilium. During ciliary assembly, IFT trains are occupied with a heavy load of tubulin. However, once cilia reach steady-state length this cargo load is dramatically reduced and IFT trains during ciliary maintenance run near empty with respect to tubulin. Our in vivo tubulin transport studies showed that the average transport frequency of GFP-tagged α-tubulin in lf2-1 cells is significantly higher than that for wild-type steady state cilia; the elevated frequency of lf2-1 full-length cilia resembles that of growing wild-type cilia. Interestingly, unlike tubulin transport in wild-type cells, a length-dependent decrease in the frequency of α-tubulin transport was not observed for lf2-1. These data suggest that
the increased length of \textit{lf2-I} cells involves the defective regulation of tubulin transport and that the LF2 protein might play a role in regulating the tubulin load on IFT particles.

LF2 encodes a CDK-like kinase that interacts with two different proteins, LF1p and LF3p, to form the length regulating complex (LRC) \cite{129}. The LRC localizes to the cell body in \textit{C. reinhardtii}. However, the function of the complex is unknown. Mutants in \textit{lf2} display cilia of variable length with some 2-3 times longer than that seen in wild-type cells \cite{127, 129}. The mammalian orthologue of LF2, CCRK, was shown to phosphorylate ICK, a MAP-kinase related to \textit{Chlamydomonas} LF4 \cite{130}. LF4 localizes to cilia and mutations in the protein result in cilia three times longer than that of wild-type cells \cite{127}. Since the LF4 kinase is located in cilia and the LF2 kinase is present in the cytoplasm this situation could allow for a direct link that would relay changes in ciliary length to a cellular mechanism controlling the loading of IFT particles. To examine the role of LF2 in cargo regulation one could start by studying aspects of tubulin transport in dikaryons.

\textit{Chlamydomonas} is an isogamous species, with two mating types (+ and -). Under nitrogen starvation gametogenesis is induced when gametes of opposite mating types interact and fuse to form one cell, known as a dikaryon. Dikaryons possess four cilia, two from the “plus” mating type cell and two from the “minus” mating type cell, along with a shared cytoplasm. The shared cytoplasm in the dikaryon allows one to analyze the \textit{in vivo} rescue of a phenotype when a mutant cell is mated to a wild-type cell. Examples include the restoration of proper ciliary length and motility when length and motility mutants are mated to wild-type strains. One could perform the following experiment to further elucidate the role that the LF2 kinase has in cargo transportation: GFP-tagged
alpha tubulin transport could be analyzed in the following zygotes \(lf2xl2\) \(\alpha\text{-tubulin-GFP},\)
\(WTxWT\) \(\alpha\text{-tubulin-GFP}\) and \(lf2\) \(\alpha\text{-tubulin-GFPxWT}\). Based on the transportation data for \(lf2-I\) cells, one could expect one of two outcomes: 1) a correction of tubulin transport from the increased levels observed in \(lf2\) cells back to the reduced levels seen in wild-type; or 2) correction of tubulin transport in the \(lf2\) cilia of the dikaryon could overshoot and the \(lf2\) cilia would show a decreased level of transport than what is seen in wild-type cilia. During the former situation, in which cargo transport is restored to normal levels, the LF2 kinase (or the LRC as a whole) would suppress extra tubulin loading when cilia are too long returning tubulin loading back to the marginal amount needed during ciliary maintenance. In the latter situation, in which the transport of tubulin overshoots the level seen in wild-type cells, the LF2 kinase would be able to actively modulate the amount of cargo loaded onto the IFT train and would adjust the level from a maintenance level to a severely reduced or non-loaded level to induce flagellar shortening. A result such as in the second scenario could indicate that the cells are able to sense alterations in ciliary length and modulate these differences through the LF2 kinase and the LRC.

While the proposed experiments above are likely to result in valuable information on the processes controlled by the LF2 kinase, they do not inform on how the kinase directly regulates these cellular processes. The identification of the substrates of the LF2 kinase requires the purification of the protein. Our lab has had success in purifying individual proteins and whole complexes through the utilization of a GFP-tag and immuno-purification with anti-GFP beads. One could apply a similar technique to purify the LF2 kinase coupled with \textit{in vitro} kinase assays with a ciliary extract; a more comprehensive approach would be to examine the phosphoproteome of \(lf2\) and wild-type
cells in search of differences between the two samples that would be identified with the use of mass spectrometry. The amount of ciliary proteins, especially tubulin that is loaded on to IFT trains is likely to have a profound impact on the overall length of the cilium. Loading of IFT trains with ciliary precursors occurs in the cell body. However, proteins vital to this process remain a mystery. The cytoplasmic localization of LF2 in conjunction with the observed defects in tubulin trafficking in these cells makes the kinase a prime candidate in regulating the cargo load placed on IFT trains.

4.3 ROLE OF IFT IN MAINTAINING THE EXCLUSIVITY OF THE CILIUM

The frequency of tubulin transport by IFT is highly upregulated during ciliary growth whereas relatively low amounts of tubulin are transported by IFT during ciliary maintenance. This pattern of regulation has been shown for other axonemal proteins as well. These results support the differential-cargo loading model of ciliary length regulation. This model states that while the quantity of IFT particles cycling through a cilium remains essentially the same regardless of growth state the occupancy of those same IFT particles with structural proteins are adjusted in a cilia-length dependent manner. More generally, while IFT particles continually translocate through cilia they are only heavily loaded with ciliary precursors during growth. IFT particles display a low occupancy during times of ciliary maintenance. This model begins to answer fundamental questions regarding how the IFT system is utilized to maintain ciliary length. A second equally interesting question is: If IFT particles are not carrying cargo during ciliary maintenance why are they running at all?
Motor activity in the cell body is regulated by cargo presence: motors are active when bound to cargo and in the absence of cargo become autoinhibited in an effort to limit wasteful ATP consumption [131]. However, for the IFT system, it is thought that the IFT particles themselves are the primary cargos of the IFT motors resulting in a constitutively active IFT transport system in cilia regardless of axonemal cargo presence. If IFT is not being used to transport proteins for assembly, then what are the benefits of running the machinery that could offset the high energy cost of active transport? One hypothesis is that IFT trains and the molecular motors involved, specifically kinesin, participate in measuring the length of the cilium. After unloading cargo at the ciliary tip, anterograde IFT trains reorganize into retrograde trains that are carried back to the base of the organelle by IFT dynein 1b. The anterograde motor, kinesin, however is not transported back by retrograde IFT but returns to the ciliary base by diffusion in C. reinhardtii. Recent work showed that the time needed for kinesin to diffuse back to the ciliary base was sufficient to generate a stable steady-state length of cilia and could therefore serve as a ruler for length measurement [132]. As the cilia lengthen, diffusion delays the return of kinesin to the basal body pool, therefore depleting the available kinesin available for anterograde IFT train formation [87]. While this hypothesis has merit we consider it unlikely. The above results suggest that the IFT system is closed – proteins from retrograde IFT trains are remodeled in a basal body pool where they are then used for anterograde IFT. However, work from our lab showed that the IFT system (with respect to IFT-A and motor proteins) is open [27]. Instead of IFT and motor proteins being reassembled and reused from a pool near the basal bodies, proteins for anterograde IFT train formation are recruited from a cytoplasmic pool and after they
cycle through cilia are returned to the cell body [27]. The pool of kinesin available for anterograde IFT trains is not dependent on return of kinesin from cilia but instead is reliant on the recruitment of the protein from the cytoplasm negating the “diffusion of kinesin as a ruler” model. Additionally, the Chlamydomonas fla3 mutant displays a significantly reduced IFT frequency - less kinesins undergoing diffusion - however the length of cilia in these cells is normal [133]. While the diffusion, and therefore, availability of kinesin might play a role in ciliary length regulation, the activity of additional proteins and signaling molecules are likely required to sense the length of the cilia and ensure proper organelle size.

Since IFT trains continually cycle through the cilium, IFT could be employed to monitor the protein composition of the organelle. While not a membrane-bound organelle, the cilium is partitioned from the cell body by the presence of the transition zone and other structural complexes. This partitioning results in a specific protein composition of the cilium that is: 1) different from that found in the cell body; and 2) vital for the proper functioning of the organelle. Additionally, the transition zone establishes concentration gradients between the cell body and the cilium. Just as many proteins are highly enriched in the cilium, many cytoplasmic proteins are effectively excluded from the organelle. While the transition zone is a restrictive barrier for many large cytoplasmic proteins, small to midsized proteins (<50 kDa) could readily pass through the structure and diffuse down their concentration gradient into cilia. An example of such a protein is phospholipase D (PLD). This small membrane associated protein is effectively excluded from cilia in control cells with 99% of the total PLD residing in the cell body [80]. However, in mutants of the BBSome, PLD displays high levels of
accumulation in cilia with only 40% of PLD localized to the cell body [80]. PLD was shown to enter the cilia of *C. reinhardtii* independently of IFT but its removal from cilia was dependent on IFT and the BBSome [80]. In this situation, cytoplasmic PLD leaks into the cilia where it is identified by the IFT/BBSome system and transported against its concentration gradient back into the cell body. The perpetual cycling of IFT trains through cilia could provide a mechanism to continuously monitor the ciliary environment. When cytoplasmic proteins leak into the cilia IFT is utilized to scavenge these proteins and actively export them against their concentration gradient to maintain the proper protein composition of the cilium.

To address the scavenging properties of the IFT system one could take advantage of a temperature sensitive mutant of the IFT pathway (*fla10*). IFT can be abolished in these cells following an incubation at the restrictive temperature. If IFT removes cytoplasmic proteins leaking into cilia then proteins should begin to accumulate in the cilia of *fla10* cells when IFT is abolished. To monitor protein accumulation, cilia from these cells can be isolated and the protein composition compared to a wild-type strain under the same conditions. Differences between the two conditions could be analyzed via mass spectrometry to identify the aberrant proteins. Utilizing a temperature-sensitive mutant of the IFT pathway is an initial step in beginning to explore the functions of IFT in maintaining the exclusivity of the cilium.
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